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PRINCIPAL INVESTIGATOR: Jonathan M. Shillingford, Ph.D.

CONTRACTING ORGANIZATION: Foundation for Advancement of Education in the Sciences, Incorporated
Bethesda, Maryland 20814-1460

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Jonathan M. Shillingford, Ph.D.

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Foundation for Advancement of Education in the Sciences, Incorporated
Bethesda, Maryland 20814-1460
E-Mail: jonshi@helix.nih.gov

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The ongoing purpose of this work is to assess the contribution of the tyrosine kinase Jak2 in the development and progression of breast cancer. Using a mammary gland transplantation approach, it has been demonstrated that Jak2 is required for pregnancy-mediated proliferation and differentiation of mammary epithelium as assessed by electron microscopy and immunohistochemical analyses. Experiments are currently underway to generate a conditional mouse model in which the Jak2 gene can be specifically inactivated in mammary epithelium. Analyses of tumors arising in a mouse model with mutations in the *Brca1* and *p53* genes have revealed that the nuclear localization of Stat5a and Stat3 is heterogeneous both within tumors and between individual tumors. To further determine the possible role of Stat5a in human breast cancer, we have utilized human tissue arrays. Similar to the mouse model examined, heterogeneous nuclear and cytoplasmic Stat5a and Stat3 were observed. Finally, we have documented for the first time that the water transporter, aquaporin 5 (AQP5) and the Na-K-Cl cotransporter, NKCC1, are highly expressed in Brca1/p53 mutant mice suggesting they may be useful as prognostic markers.

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INTRODUCTION

There is a large body of evidence in the literature demonstrating that the constitutive activation of signal transducers and activator of transcription (Stats) is a common feature of cancer cell lines and primary tumors and is responsible for hyperproliferation. However, the nature of the upstream events involved in the initial activation of these Stat proteins has received comparatively little attention. It has been demonstrated that members of the Jak kinase family are the primary mediators of Stat activation and Jak fusion proteins result in the development of cancerous lesions. The ongoing objective of the proposal is to ascertain whether the tyrosine kinase, Jak2, contributes to constitutive activation of Stats leading to the development and/or progression of breast cancer. To ascertain the role of Jak2 in tumor formation, it was first necessary to understand the role of Jak2 in normal mammary gland development. Using a mammary gland transplantation approach, we have firmly established that Jak2 is the central kinase involved in pregnancy-mediated differentiation of mammary epithelium. Furthermore, similar experiments have revealed that the upstream prolactin receptor and downstream transcription factor, Stat5, are both required for functional differentiation of mammary epithelium during pregnancy. Utilizing a mouse model that carries mutations in the two genes *Brca1* and *p53*, we have characterized the mammary tumors arising in these mice and assessed the activation status of Stat5. Finally, we have utilized human tissue arrays to ascertain whether Stat activation is a general feature of human breast cancer.

BODY

Analysis of Jak2/Stat5 protein in normal mammary gland and mammary tumors

As reported previously, the Jak2 antibodies currently available commercially work well in cell culture systems, as evidenced by numerous publications, but have not been ideal for use in the analysis of Jak2 protein in whole tissue extracts prepared from mammary gland. Several Jak2 antibodies from different sources and a phospho-specific Jak2 antibody have been used to examine the status of Jak2 protein during normal mammary gland development. While injection of prolactin results in nuclear accumulation of Stat5a that can be readily detected by immunofluorescence, the detection of activated Jak2 has not so far been possible. Based on the lack of proliferation and functional differentiation in the absence of Jak2, coupled with the known activation profile of Stat5a, it is hypothesized that Jak2 is activated in mid-pregnancy and is the primary upstream mediator of Stat5a phosphorylation and subsequent target gene activation. Although it has not been possible to demonstrate the activation status of Jak2 in the current system, constitutive activation of Jak2/Stat5 signaling (1) and the generation of constitutively active Jak2 fusion proteins (2, 3) has been shown to result in leukemia. Furthermore, experiments have demonstrated that the Jak2 inhibitor, AG490, halts the growth of certain human B-precursor leukemic cells (4). Thus the current data strongly suggest that Jak2 contributes to cancer formation and progression.

The activation profile of Stat5 suggests it is associated with the differentiation phase of epithelial development (5), which has recently been confirmed experimentally (see 2, published papers). Since Stat5a is a downstream mediator of Jak2 activity, we have determined the localization of Stat5a in tumors isolated from mice carrying

mutations in the *Brca1* and *p53* genes. These mice reliably develop numerous mammary tumors in the space of 6 – 8 months (6) and a panel of mouse and human pathologists recently agreed that the histopathology of tumors arising in these mice is very similar to human breast cancers exhibiting Brca1/p53 mutations. Since it has previously been reported that Stat3 is constitutively phosphorylated in human breast cancer cell lines, the localization of Stat3 was also determined. Using an antibody specific for the Stat5a isoform, several tumors derived from *Brca1/p53* mutant mice were analyzed (Fig. 1). In mature animals (5 – 6 months old) that were tumor free, significant nuclear Stat5a staining (Fig. 1A and B, white arrows) was evident in some cells while others lacked detectable Stat5a (Fig. 1A and B, yellow arrows and Fig 1C). Analysis of tumor samples revealed nuclear Stat5a in some cells (Fig. 1G – I, white arrows) but not others (Fig. 1G – I, yellow arrows), and some tumors contained no detectable Stat5a (Fig. 1D – F). These data suggest there is considerable heterogeneity both within and between tumors. In tumor 1 (Fig. 1D – F), a gradual loss of the epithelial cell-cell contact marker E-cadherin is observed in various parts of the tumor indicative of a loss of functional differentiation. Thus it is possible that the accumulation of nuclear Stat5a and consequential hyperproliferation occurs early in tumor development when the tumor retains a certain degree of functional differentiation. As the tumor progresses, nuclear Stat5a, E-cadherin and other markers of functional differentiation are lost as the tumor becomes less differentiated. Analysis of Stat3 localization (Fig. 2) revealed tumors with no detectable Stat3 (Fig. 2G – I) and tumors with distinct foci of nuclear Stat3 (Fig. 2E and F, white arrows).

While these data suggest that Stat proteins may be involved in mammary tumor development in mice, they do not address whether this situation may be true of human breast cancers. Therefore, to extend these observations further, human tissue array slides were obtained from the Tissue Array Research Program (TARP). These slides consist of arrayed tissue punches derived from normal tissue and tumor tissue. Despite limited clinical information, these arrays have permitted the immunohistochemical assessment of Stat5a and Stat3 proteins. As seen in Fig. 3, heterogeneity in Stat3 and Stat5a staining was observed. Thus some tumors possessed cells with nuclear Stat3 and Stat5a (Fig. 3A and B, white arrows), others nuclear Stat3 (Fig. 3C and E, white arrows) and cytoplasmic Stat5a (Fig. 3D and F, orange arrows), and others with no detectable Stat3 or Stat5a (Fig. 3G and H). These data highlight the heterogeneity of Stat activation status in human breast cancer. However, since the normal control human mammary epithelium was not apparent on the slides it is difficult to draw firm conclusions from these data. Commercial human tissue arrays are now available that contain concise clinical information and histopathlology. These will be used to assess more accurately the extent of Stat5a/Stat3 activation in normal vs. tumorigenic breast cancers.

Figure 1: Localization of Stat5a in tumors derived from *Brca1/p53* mice.

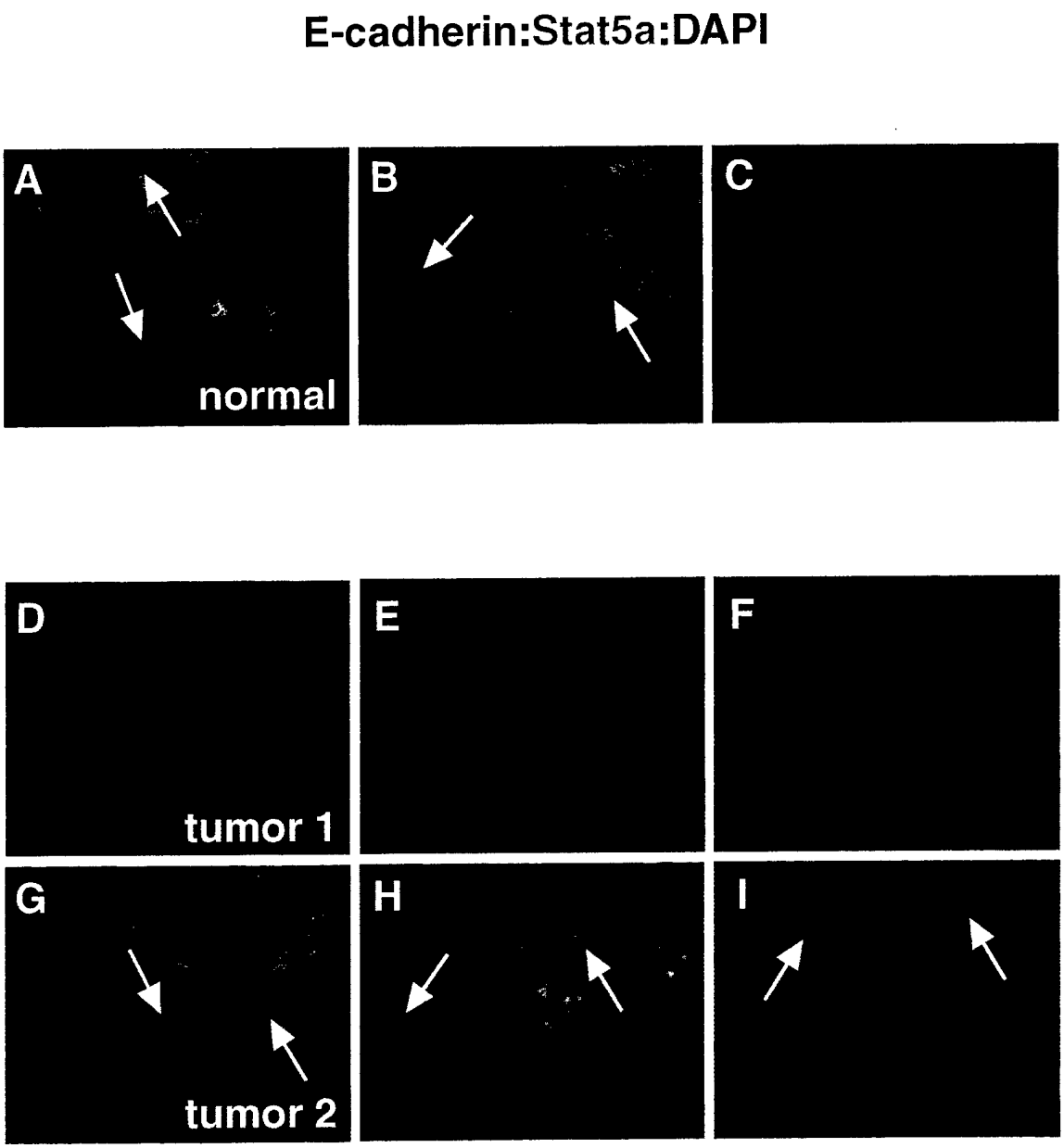


Figure 1: Tumorigenic and non-tumorigenic mammary tissue was isolated from mature female *Brca1/p53* mice, fixed, embedded in paraffin and sectioned at a thickness of 5 μm. Sections were incubated with antibodies to Stat5a and E-cadherin, which were subsequently visualized with fluorescent-conjugated secondary antibodies (Stat5a, green; E-cadherin, red). Nuclei were visualized with DAPI (blue). Nuclear Stat5a was observed in normal epithelium (A and B, white arrows). E-cadherin was progressively lost in tumor 1 (D – F) and nuclear Stat5a was observed in tumor 2 (G – I, white arrows).

Figure 2: Localization of Stat3 in tumors derived from *Brca1/p53* mice.

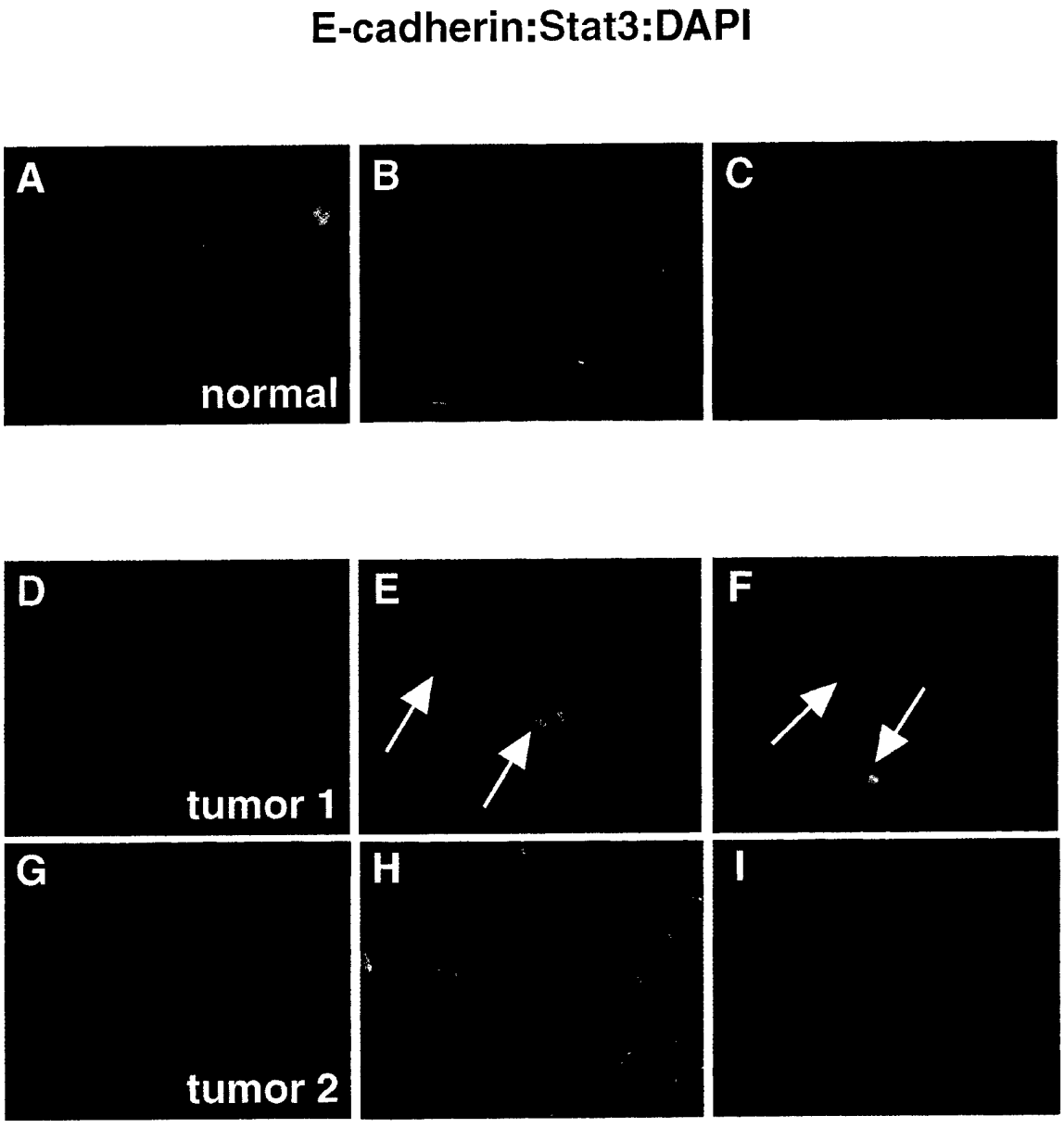


Figure 2: Tumorigenic and non-tumorigenic mammary tissue was isolated from mature female *Brca1/p53* mice, fixed, embedded in paraffin and sectioned at a thickness of 5 μm. Sections were incubated with antibodies to Stat3 and E-cadherin, which were subsequently visualized with fluorescent-conjugated secondary antibodies (Stat3, green; E-cadherin, red). Nuclei were visualized with DAPI (blue). Stat3 was not detected in normal epithelium (A – C). Note that tumor 1 had foci of nuclear Stat3-positive cells (E and F, white arrows) and a progressive loss of E-cadherin was observed in tumor 2 (G – I).

Figure 3: Localization of Stat3 and Stat5a in human breast cancer samples.

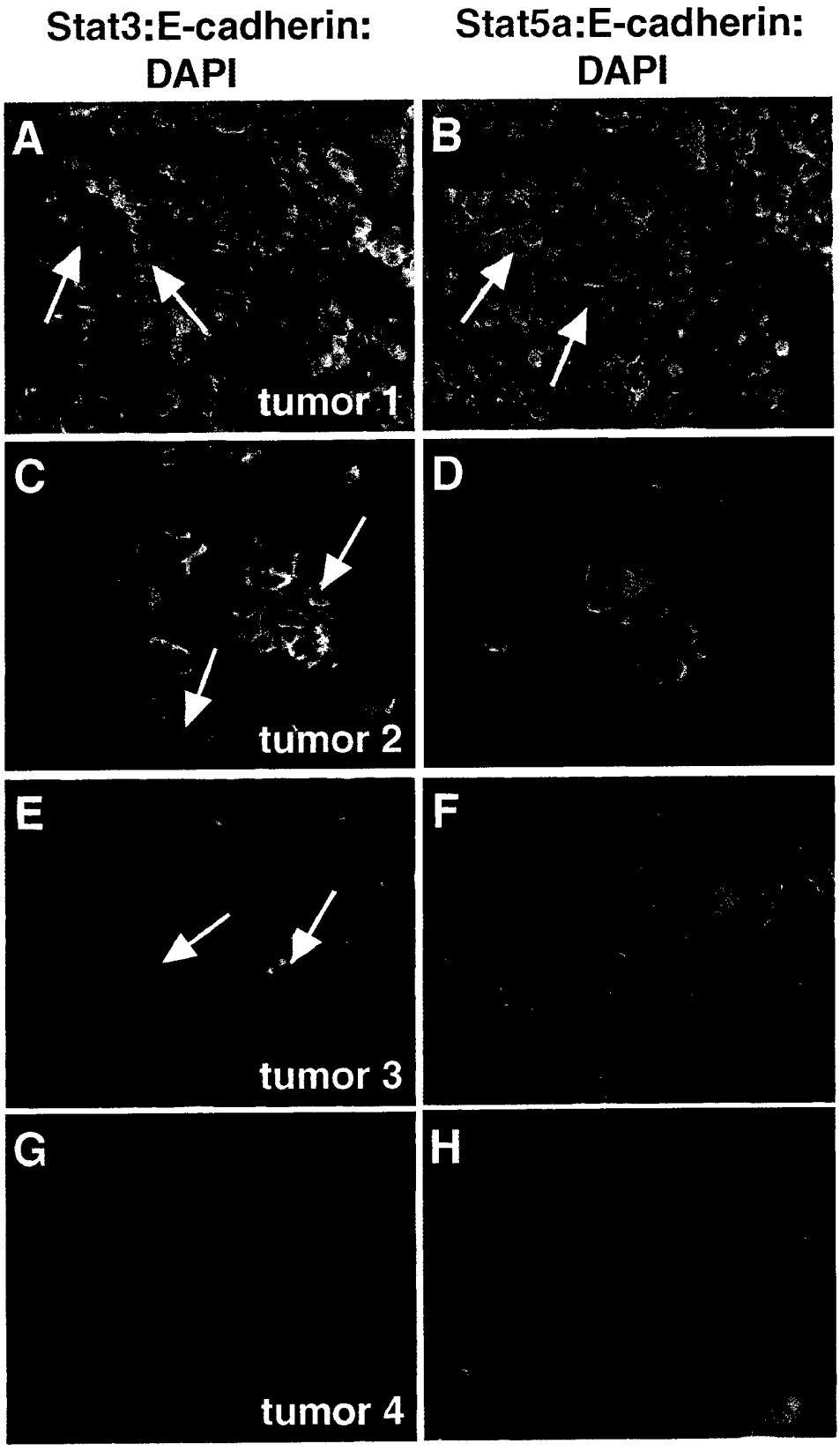


Figure 3: Tissue array slides were incubated with antibodies to Stat3 and E-cadherin (A, C, E and G) or Stat5a and E-cadherin (B, D, F and H), which were subsequently visualized with fluorescent-conjugated secondary antibodies (Stat3 and Stat5a, green; E-cadherin, red). Total nuclei were visualized with DAPI (blue). Nuclear Stat3 (A, white arrow) and Stat5a (B, white arrow) were observed in human breast tumor 1; nuclear Stat3 (C and E, white arrows) and cytoplasmic Stat5a (D and F, orange arrows) were observed in human breast tumors 2 and 3. No detectable Stat3 or Stat5a protein was detectable in human breast tumor 4 (G and H).

Jak2 embryonic transplants

Using a mammary transplantation approach, we have demonstrated that Jak2 is the central tyrosine kinase responsible for the pregnancy-mediated differentiation of mammary epithelium (see 1, published papers). Since Stat5 is a downstream mediator of Jak2 kinase activity and transplantation studies have determined that mammary epithelium fails to undergo differentiation in the absence of Stat5, the subcellular localization of Stat5a was determined in wild type and Jak2-null epithelium at pregnancy day 13 and parturition by immunohistochemistry using a Stat5a-specific antibody (Fig. 4). These experiments revealed the presence of nuclear Stat5a at pregnancy day 13 (Fig. 4A, white arrowheads) and parturition (Fig. 4C, white arrowheads) in wild type epithelial cells. In contrast, far fewer Jak2-null epithelial cells exhibited nuclear Stat5a localization at pregnancy day 13 and parturition (Fig. D and E, white arrowheads). In addition, some cells had evidence of cytoplasmic but no nuclear Stat5a staining (Fig. 4D, yellow arrows) and still others had no evidence of either cytoplasmic or nuclear Stat5a (Fig. 4B and D, white arrows). Quantitation of the percentage of cells positive for nuclear Stat5 vs. the number of propidium iodide-positive cells revealed that while 70 – 80% of wild type cells possessed nuclear Stat5a, only 20% of Jak2-null cells had detectable nuclear Stat5a (Fig 4E). These results demonstrate that Jak2 activity is required for efficient and robust Stat5a phosphorylation and subsequent translocation to the nucleus. Since 20% of the cells exhibit nuclear Stat5a it is apparent that Jak2-independent phosphorylation of Stat5a also occurs and further suggests that a threshold of nuclear Stat5a needs to be attained to elicit functional differentiation that is not achieved in the absence of Jak2.

To determine the proliferative capacity of Jak2-null epithelium, transplanted mice were injected with estrogen and progesterone to simulate the hormonal environment experienced during pregnancy. Subsequently, proliferating cells were labeled by injecting BrdU and BrdU incorporation was assessed by immunohistochemistry (Fig. 5A and B). While transplanted wild type epithelium responded normally to hormonal injection and demonstrated a BrdU incorporation of approximately 10% (Fig. 5A and C), the proliferative capacity of Jak2-null epithelium was reduced by 95% (Fig. 5B and C). These data demonstrate that Jak2 mediates proliferation as well as differentiation of mammary epithelial cells during pregnancy, most likely through target genes activated by Stat5a.

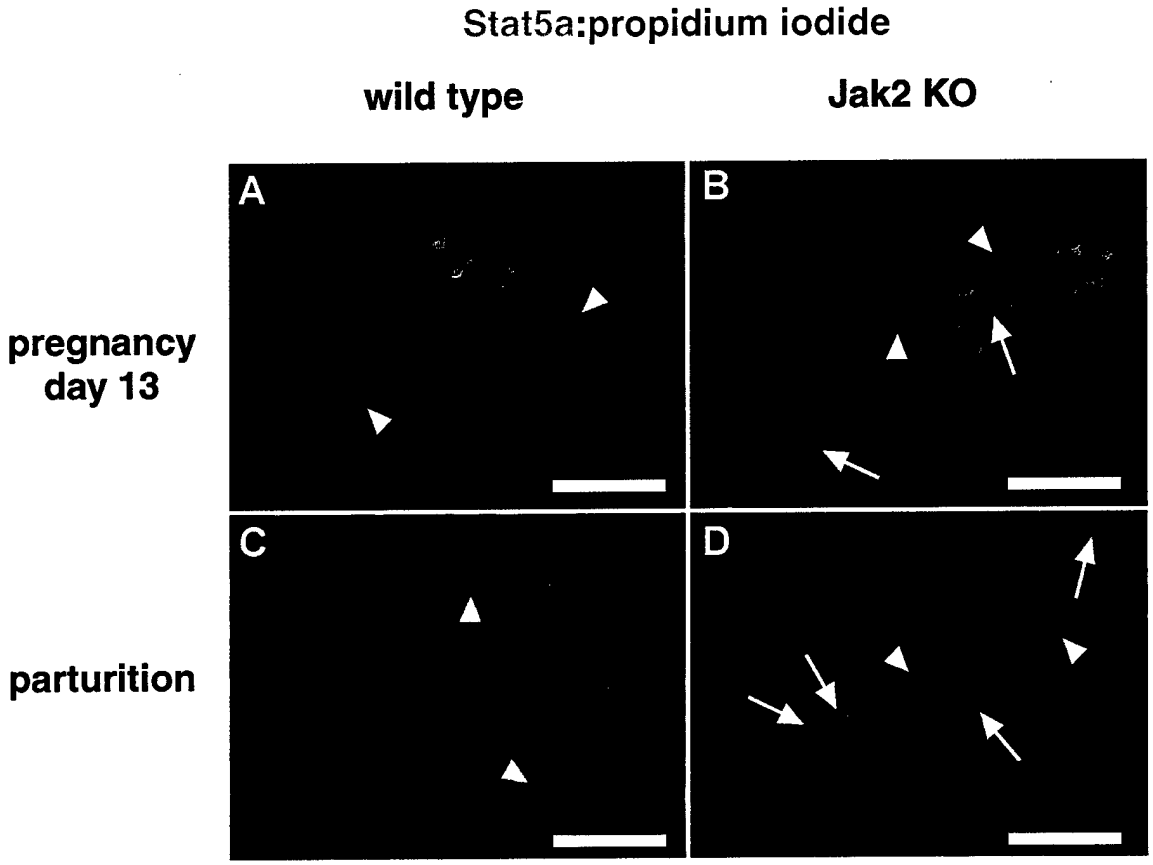
Histological evaluation of Jak2-null epithelium at pregnancy and parturition revealed an apparent lack of functional differentiation as evidenced by the persistence of large areas of adipocytes and a failure of the epithelium to form alveolar structures. The ductal nature of these structures was confirmed experimentally through the immunofluorescence analysis of two proteins; NKCC1 (a ductal cell marker) and Npt2b (a secretory cell marker). In the absence of Jak2, there was maintenance of a high level of NKCC1 and a lack of induction of Npt2b. We have identified and utilized another protein marker, aquaporin 5 (AQP5), which is exclusively expressed in the apical membrane of mammary ductal cells in the virgin animal (Fig. 6A) but absent at parturition (Fig. 6B). Utilizing immunofluorescence analyses, we have further determined that the epithelial structures present in Jak2-null transplants at parturition retain ductal features as evidenced by the persistence of apical AQP5 (Fig. 6D). In contrast, virgin Jak2-null epithelium appeared normal with respect to AQP5 protein (cf. Fig. 6A and C).

To further understand the undifferentiated nature of Jak2-null epithelium on the ultrastructural level, electron microscopy was performed (Fig. 7). In normal wild type epithelium at parturition (Fig. 7A) there was evidence of large, single-cell layered alveolar structures containing large lipid droplets and casein micelles. Extensive rough endoplasmic reticulum and active Golgi apparatus was present in the epithelial cells, providing morphological evidence for the attainment of secretory function. In contrast, Jak2-null epithelium (Fig. 7B) appeared disorganized and the epithelial structures were composed of several layers of epithelial cells. There was no evidence of prominent smooth endoplasmic reticulum or active Golgi apparatus and small lipid droplets were present near the basolateral membrane suggestive of an overall defect in the normal vectorial secretion of lipid. These data establish on an ultrastructural level that Jak2 kinase activity is required for the normal differentiation of mammary epithelium at pregnancy and the formation of active alveolar structures.

Conditional targeting and deletion of the Jak2 gene

Southern blots have been performed on genomic DNA isolated from targeted, FIAU-resistant ES cell clones. Since the amount of DNA recovered was limited and did not permit the successful analysis of targeting, ES cell clones were expanded a second time to facilitate the isolation of more genomic DNA for analysis. A total of 5 clones have since been identified that contain a putative targeting event based on Southern analysis. These clones are currently being amplified for injection into blastocysts, which will subsequently be transplanted into pseudo-pregnant mice.

Figure 4: Reduced nuclear localization of Stat5a in the absence of Jak2.



E

Nuclear Stat5a vs. total number of luminal epithelial cells

	Jak2-null	wild type
Pregnancy day 13 A	26.8 ± 2.1 %	71.2 ± 1.5 %
Pregnancy day 13 B	23.1 ± 1.1 %	74.8 ± 2.8 %
Parturition A	19.6 ± 1.4 %	79.7 ± 2.1 %
Parturition B	25.7 ± 2.0 %	79.9 ± 2.2 %

Figure 4: Transplanted wild type (A and C) and Jak2-null (B and D) mammary glands were isolated from mice at pregnancy day 13 (A and B) and parturition (C and D). Tissue was fixed, embedded in paraffin, sectioned at a thickness of 5 μm and processed for immunohistochemistry. Anti-Stat5a antibody was applied to the sections and subsequently visualized with a fluorescent-conjugated secondary antibody (green). Nuclei were visualized with propidium iodide (red). Note the nuclear localization of Stat5a in wild type transplants (A and B, white arrowheads). In Jak2-null transplants, there was evidence of cells exhibiting nuclear Stat5a (B and D, white arrowheads), cytoplasmic Stat5a (D, yellow arrows), and no detectable Stat5a (B and D, white arrows). The proportion of cells containing nuclear Stat5a vs. the total number of propidium iodide-positive cells was calculated and counts are represented in (E).

Figure 5: Reduced proliferation of Jak2-null epithelium upon hormone stimulation.

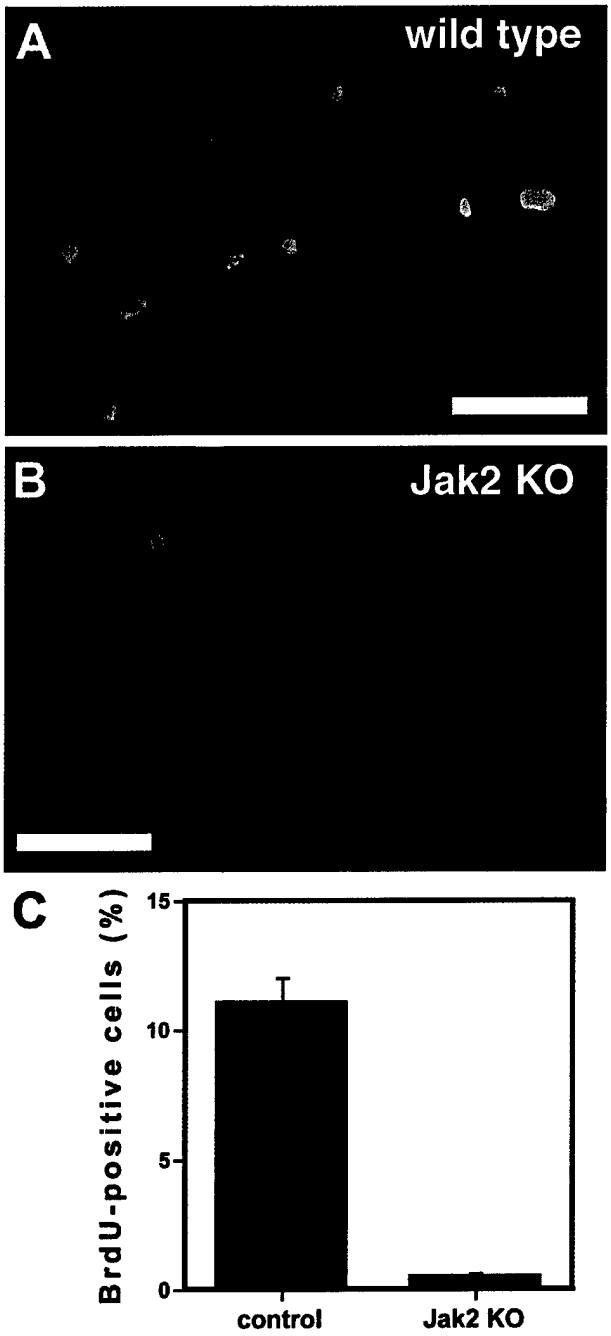


Figure 5: Nine-week-old transplanted mice were treated with a single injection of estrogen and progesterone for 48 h. Two hours prior to sacrifice, mice were injected with BrdU. The mammary glands were subsequently isolated and tissue was fixed, embedded in paraffin, sectioned at a thickness of 5 μm and processed for immunohistochemistry. Anti-BrdU antibody was applied to the sections and subsequently visualized with a fluorescent-conjugated secondary antibody (green). Nuclei were visualized with DAPI (blue). Sections representing wild type (A) and Jak2-null epithelium (B) are shown. Quantiation of the number of BrdU-positive cells was performed (C). Note that the BrdU-positive cell in (B) represents one of only a few BrdU-positive cells within the entire Jak2-null gland.

Figure 6: Evaluation of AQP5 protein in Jak2-null epithelium.

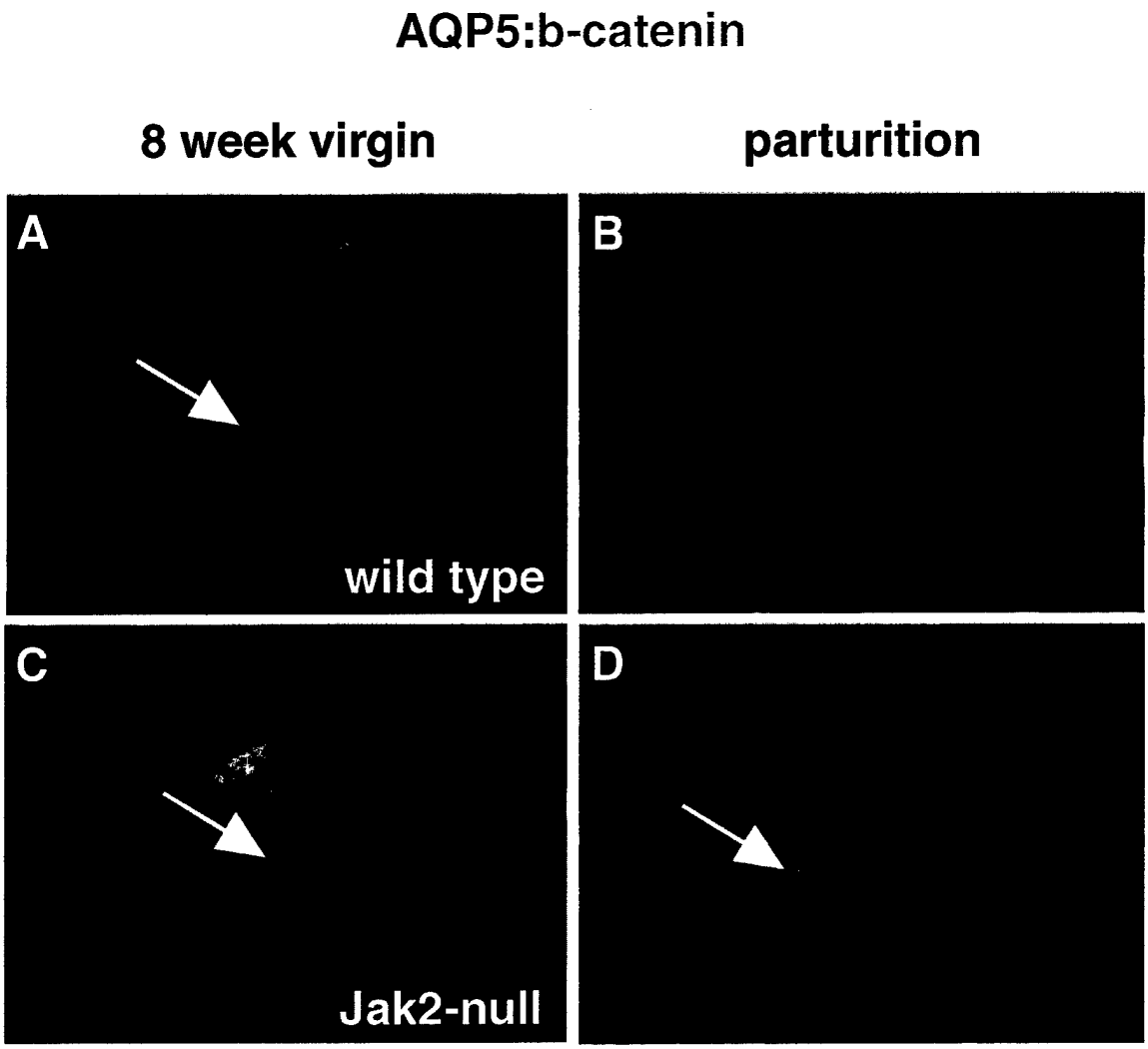
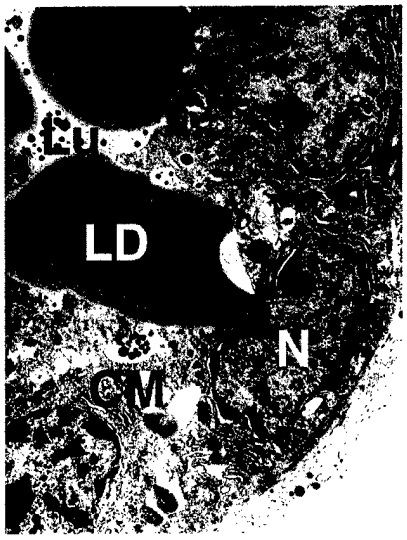


Figure 6: Transplanted wild type (A and B) and Jak2-null (C and D) mammary glands were isolated from 8-week-old virgin mice (A and C) or mice at parturition (B and D). Tissue was fixed, embedded in paraffin, sectioned at a thickness of 5 μm and processed for immunohistochemistry. Anti-AQP5 and anti-beta-catenin antibodies were applied to the sections and subsequently visualized with fluorescent-conjugated secondary antibodies (AQP5, red; beta-catenin, green). Note the apical localization of AQP5 protein in the virgin ductal epithelium in wild type (A, white arrow) and Jak2-null (C, white arrow) samples. In contrast to the absence of detectable apical AQP5 protein in wild type epithelium at parturition (B), Jak2-null epithelium exhibited a persistence of AQP5 protein (D) similar to that observed in virgin samples (A and C).

Figure 7: Ultrastructural evaluation of Jak2-null epithelium at parturition.

A) wild type



B) Jak2-null

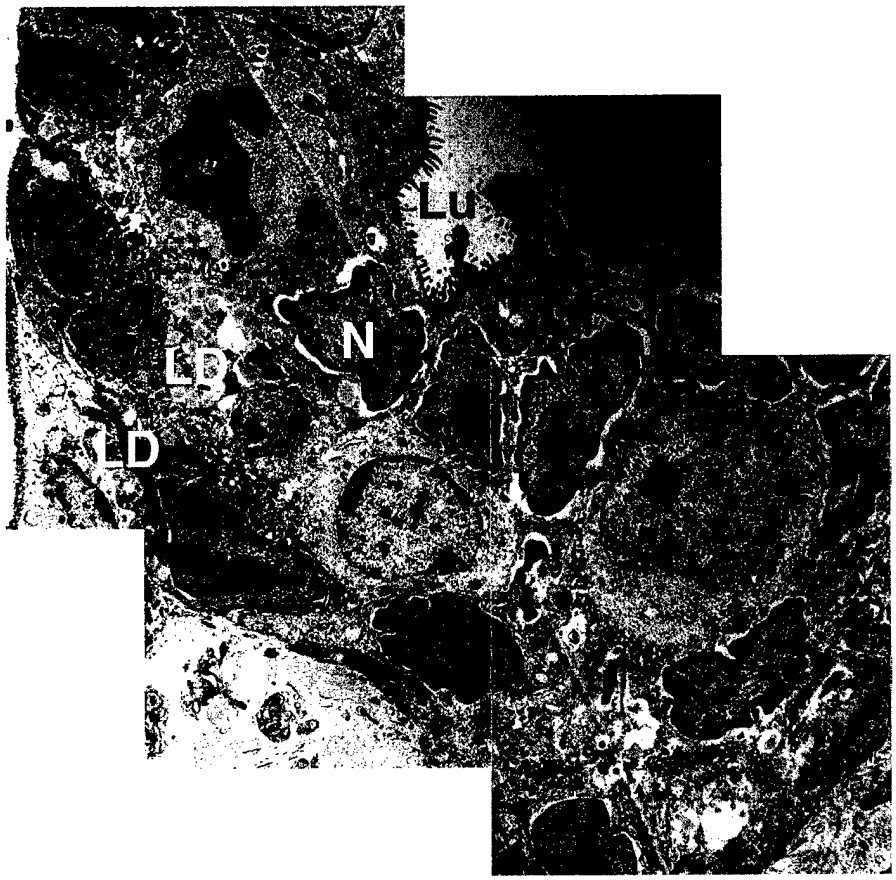


Figure 7: Transplanted wild type (A) and Jak2-null (B) mammary epithelium was isolated from mice at parturition and processed for electron microscopy. Note the presence in (A) of a single layer of epithelial cells surrounding a large lumen (Lu) containing numerous lipid droplets (LD) and casein micelles (CM). In contrast, Jak2-null epithelium (B) has several layers of epithelial cells, a small lumen (Lu) and evidence of small lipid droplets (LD) at the basolateral membrane. N = nucleus.

Figure 8: Localization of AQP5 and NKCC1 in tumors derived from *Brca1/p53* mice.

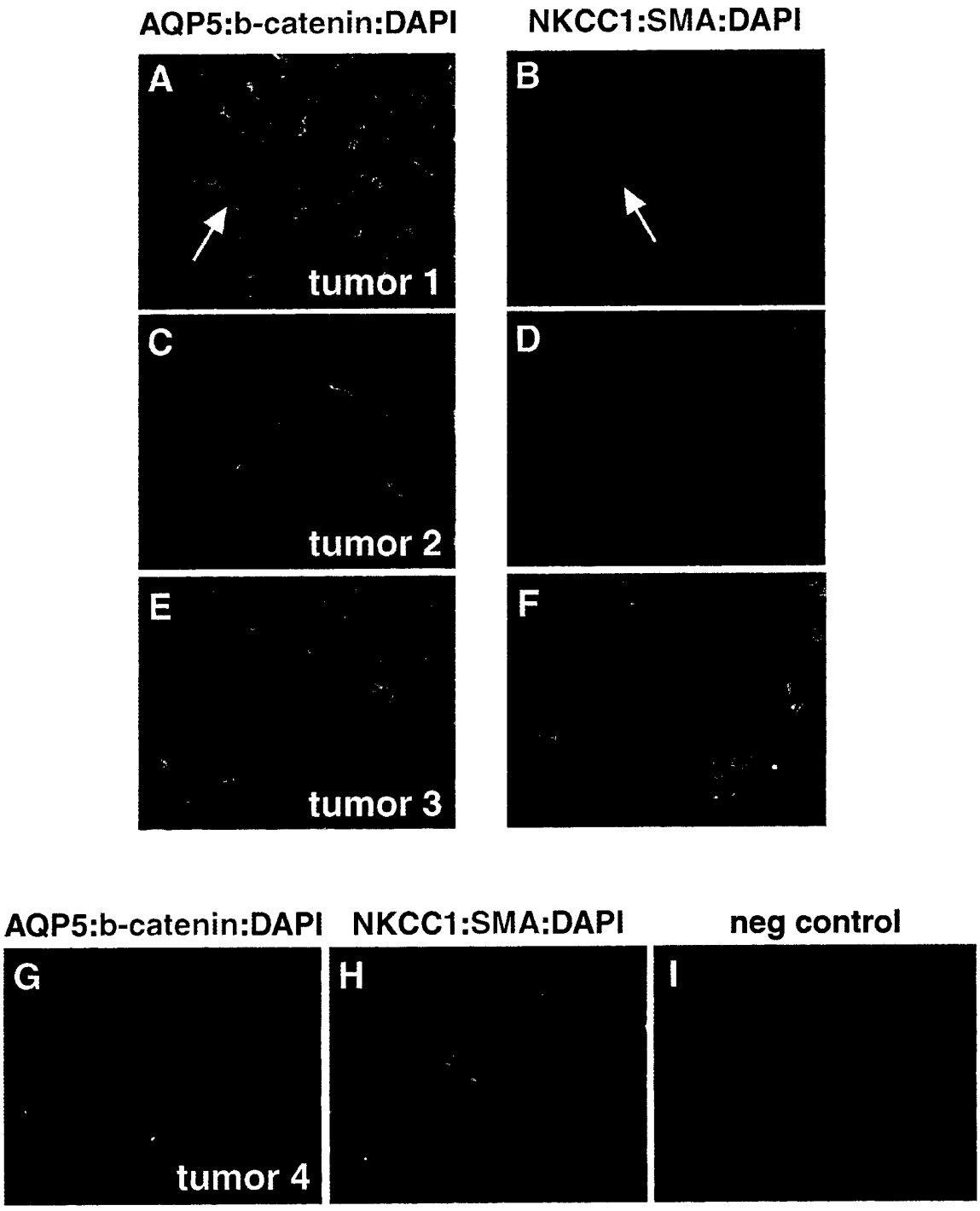


Figure 8: Tumorigenic mammary tissue was isolated from mature female *Brca1/p53* mice, fixed, embedded in paraffin and sectioned at a thickness of 5 μm. Sections were incubated with antibodies raised against AQP5 and beta-catenin (A, C, E and G) or NKCC1 and smooth muscle actin (SMA) (B, D, F, H), which were subsequently visualized with fluorescent-conjugated secondary antibodies (AQP5 and NKCC1, red; beta-catenin and SMA, green). Nuclei were stained with DAPI (blue). AQP5 protein was localized at discrete borders between distinct clusters of tumor cells (A, white arrow) while NKCC1 was present around the entire membrane of individual tumor cells (B, yellow arrow). Both AQP5 and NKCC1 were readily detected in three tumors (tumor 1, 2 and 3) but absent from a fourth tumor (tumor 4).

KEY RESEARCH ACCOMPLISHMENTS

- Evidence of nuclear Stat5a and Stat3 in tumors derived from *Brca1/p53* mouse mammary tumors.
- Evidence of nuclear Stat5a and Stat3 in human breast cancers samples.
- Establishment of AQP5 as a marker of virgin ductal epithelial cells.
- Evidence that AQP5 and NKCC1 are makers of mammary tumors arising in *Brca1/p53* mice.

REPORTABLE OUTCOMES

Published papers:

1. **Shillingford, JM**, Miyoshi, K, Robinson, GW, Grimm, SL, Rosen, JM, Neubauer, H, Pfeffer, K and Hennighausen, L (2002). Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium. *Molecular Endocrinology* 16:563-570.

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CONCLUSIONS

The ongoing proposal has further defined the role of Jak2 in normal mammary gland development and assessed the activation status of its downstream effector, Stat5a. Through the use of immunohistochemistry, the localization of Stat5a and Stat3 in mouse mammary tumors arising in *Brca1/p53* mutant mice has been determined. These experiments have been extended to human tissue and the analysis of Stat5a and Stat3 in human breast cancer samples has been assessed. These studies demonstrate that the cellular localization of Stats is heterogeneous both within individual tumors and between tumors and likely reflects their differentiation status. Further assessment of mouse mammary tumor samples reveals that AQP5 and NKCC1 are highly expressed in some clusters of tumorigenic cells, suggesting that these proteins may be useful markers of tumor progression. Ongoing work includes the generation of a Jak2 conditional knockout mouse model to assess the contribution of the Jak signaling pathway in tumor formation and progression and the use of tissue arrays to further determine the Stat activation status in human breast tumor samples.

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Jak2 Is an Essential Tyrosine Kinase Involved in Pregnancy-Mediated Development of Mammary Secretory Epithelium

JONATHAN M. SHILLINGFORD, KEIKO MIYOSHI, GERTRAUD W. ROBINSON, SANDRA L. GRIMM, JEFFREY M. ROSEN, HANS NEUBAUER, KLAUS PFEFFER, AND LOTHAR HENNIGHAUSEN

Laboratory of Genetics and Physiology (J.M.S., K.M., G.W.R., L.H.), National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892; Department of Molecular and Cellular Biology (S.L.G., J.M.R.), Baylor College of Medicine; Houston, Texas 77030; and Institute of Medical Microbiology (H.N., K.P.), Immunology, and Hygiene, Technical University of Munich, D-81675 Munich, Germany

The PRL receptor (PrlR) and the signal transducer and activator of transcription 5a (Stat5a) are essential for the proliferation and differentiation of mammary epithelium during pregnancy. Based on tissue culture cell experiments, Jak2 is the tyrosine kinase responsible for the phosphorylation of both the PrlR and Stat5. We have now used a genetic approach to test the role of Jak2 in the mammary gland, a PrlR-responsive tissue. Because Jak2-null embryos die at E12.5, we transplanted Jak2-null mammary anlagen into cleared fat pads of wild-type mice and investigated epithelial development during pregnancy. In the absence of Jak2, no secretory alveoli were present at parturition, and epithelial cell proliferation was reduced by 95% after an acute hormone treatment. Furthermore, the Na-K-Cl cotransporter, a ductal marker, was maintained in Jak2-null epithelium and the sodium-phosphate cotransporter type IIb, a secretory cell marker, was absent. Nuclear Stat5a was only observed in a few epithelial cells in Jak2-null glands at pregnancy and parturition compared with most epithelial cells in wild-type glands. Taken together, our results demonstrate that Jak2 is a critical tyrosine kinase that conveys intracellular signals necessary for proliferation and differentiation of mammary epithelium during pregnancy. (*Molecular Endocrinology* 16: 563–570, 2002)

MAMMARY EPITHELIUM PROLIFERATES and differentiates during pregnancy through the combined action of growth factors and steroid hormones. In particular, the Prl-Stat5 pathway (where Stat5 refers to both Stat5a and Stat5b) plays a central role in alveolar development. Analyses of gene knockout mice have demonstrated that the PRL receptor (PrlR) (1) and the transcription factor, Stat5a (2), are required for functional lobuloalveolar development during pregnancy. Inactivation of the PrlR results in the inability of the alveolar compartment to proliferate and differentiate during pregnancy (1, 3, 4). Similarly, in the absence of Stat5a, alveoli fail to fully develop and differentiate (2). In contrast, inactivation of Stat5b does not significantly impair alveolar epithelial development during pregnancy (5), which can be attributed to the lower levels of Stat5b protein (6) and the ability of Stat5a to functionally compensate for the lack of Stat5b. However, we have recently demonstrated that both Stat5a and Stat5b are required for full development of the alveolar epithelium (7).

Strong Stat5 phosphorylation is detected in the mammary gland around midpregnancy and persists throughout lactation but is rapidly lost at involution (6). Because the PrlR lacks intrinsic kinase activity (8), association with a kinase is required to elicit receptor activation and subsequent activation of Stat5. The PrlR activates Jak2 in mammary gland explants *in vitro* (9), and Jak2 is responsible for PrlR phosphorylation and subsequent Stat5 recruitment and activation in tissue culture cells (*e.g.* 10–13). However, a definitive *in vivo* role for Jak2 in the mammary gland has not been established.

Functional inactivation of the *Jak2* gene has determined its essential role in erythropoiesis in the liver during early embryonic development (14, 15). Because deletion of the *Jak2* gene results in embryonic lethality (E12.5), we have used mammary gland transplantation to study Jak2-null epithelial development in a wild-type host. Using this approach, we were able to address specifically whether Jak2 is required for functional development of the mammary epithelium during pregnancy, or if other kinases are capable of compensating for the loss of Jak2.

Abbreviations: BrdU, Bromodeoxy uridine; FITC, fluorescein isothiocyanate; NKCC1, Na-K-Cl cotransporter; Npt2b, sodium-phosphate cotransporter type IIb; PrlR, PRL receptor; Stat5a, activator of transcription 5a; TRITC, tetramethyl rhodamine isothiocyanate.

RESULTS AND DISCUSSION

Lack of Alveolar Development in the Absence of Jak2

Inactivation of the *Jak2* gene leads to embryonic lethality around d 12.5 due to a lack of definitive eryth-

ropoiesis in the fetal liver (Fig. 1A). Therefore, to assess the role of *Jak2* in the development of the adult mammary gland, a transplantation approach was used. Thus embryonic mammary anlagen isolated from d 12.5 embryos (C57BL/6 genetic background) (Fig. 1, A and B) were transplanted into the epithelial-free mammary fat pads of athymic (nu/nu) nude mice, and mammary epithelial cell development was subsequently evaluated. Although athymic nude mice have reduced systemic levels of estrogen and progesterone (16), the transplantation of wild-type and Jak2-null embryonic mammary glands within the same nude mouse allowed accurate comparisons to be made.

Whole mount analyses of virgin mice carrying wild-type (Fig. 1, C and E) and Jak2-null (Fig. 1, D and F) transplants revealed no significant differences. The Jak2-null epithelium completely filled the fat pad (Fig. 1D), and there was evidence of normal terminal ductal structures (see Fig. 1, E and F, *arrowheads*). In contrast, Jak2-null transplanted tissue harvested at par-

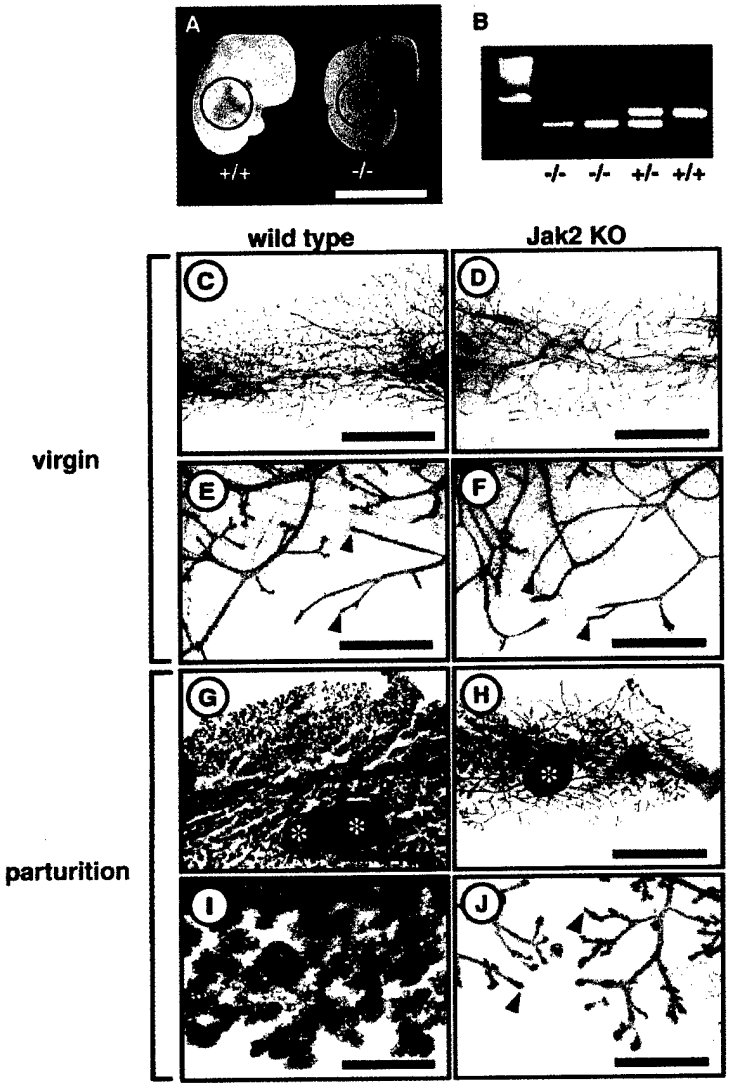

Fig. 1. Jak2 Is Essential for Alveolar Development in the Mammary Gland

A, Wild-type d 12.5 embryo (*left*) demonstrating erythropoesis in the liver (*circle*). Note the absence of erythropoesis in the liver (*circle*) of a Jak2-null embryo (*right*). B, PCR verification of embryonic Jak2 genotypes. C–F, Development of ductal epithelium in virgin wild-type (C and E) and Jak2-null (D and F) transplanted hosts at 8 wk. Normal terminal ducts (E and F, *black arrowheads*) are apparent in both glands and no significant differences in overall development are observed. G–J, Alveolar development in wild-type (G and I) and Jak2-null (H and J) transplanted hosts at parturition. The epithelium completely occupies the fat pad in wild-type epithelium (G) and numerous alveolar structures are observed (I). No evidence of distinct alveolar structures are detected in Jak2-null epithelium (H) and the persistence of terminal duct structures is apparent (J, *black arrowheads*). The original embryonic transplants are depicted (G and H, *white asterisks*). A, *Bar*, 4 mm. C, D, G and H, *Bar*, 5 mm. E, F, I and J, *Bar*, 1 mm.

turition revealed a lack of identifiable alveolar structures (Fig. 1, H and J) compared with wild-type transplants (Fig. 1, G and I). The ductal tree was maintained similar to that observed in virgin glands (see Fig. 1, J and F) and terminal ductal structures were evident (Fig. 1J, *arrowheads*).

To further analyze the morphology, histological sections were prepared. Similar to the whole mount analyses, hematoxylin and eosin-stained sections demonstrated that the ducts in virgin wild-type transplants (Fig. 2A) and Jak2-null transplants (Fig. 2B) were comparable. At parturition, the wild-type transplants contained large alveoli (Fig. 2C) with expanded lumina (Fig. 2C, *asterisks*). The alveolar cells were actively secreting milk as judged from the presence of large lipid droplets in the cytoplasm (Fig. 2C, *arrowheads*). In contrast, there was no histological evidence of lipid droplets in the Jak2-null transplants (Fig. 2D), and a persistence of fat cells was apparent. Although alveoli-like structures were present (Fig. 2D, *arrow*), no secretory alveoli were observed. These data demonstrate that Jak2 is absolutely required for the proliferation and/or differentiation of mammary secretory cells during pregnancy.

Reduced Proliferation in Jak2-Null Epithelium

To examine whether a decrease in proliferation was responsible for the lack of epithelium in Jak2-null transplants at parturition, the proliferative capacity of Jak2-null epithelium was assessed. Transplanted virgin mice (9-wk-old) were stimulated with estrogen and progesterone for 48 h followed by bromodeoxy uridine (BrdU) injection. The incorporation of BrdU was subsequently examined using immunofluorescence.

Remarkably, proliferation of Jak2-null ductal cells was reduced to 5% ($P < 0.001$) of wild-type values (Fig. 3), indicating that Jak2 is required to elicit a hormonal-induced proliferative response in mammary epithelial cells. Furthermore, this demonstrates that proliferative responses in the mammary gland are largely mediated through Jak2-dependent mechanisms. Interestingly, proliferation in PrlR-null mammary cells was 30% of that observed in wild-type cells (7). This suggests that PrlR-independent signaling pathways mediate proliferative responses in the mammary gland. This could include signals emanating from the epidermal growth factor and GH receptors, which can activate Stat5 (4), and the glucocorticoid receptor, which can synergize with Stat5 (17). Indeed, recent data have demonstrated that GH, presumably via Jak2, can induce Stat5 phosphorylation in mammary stromal cells and epithelial cells (4). Although IGF-1 plays a significant role in mammary gland development (18) and is capable of activating Jak2 in NIH3T3 cells (19), it remains to be established whether Jak2 can mediate IGF-1 actions in the mammary gland.

Unfortunately, due to the fact that nuclear Stat5 protein is detectable in the Stat5-null mammary gland, consistent with the activation of a truncated Stat5 protein (unpublished data), we were unable to accurately compare proliferative responses in Jak2-null cells *vs.* Stat5-null cells.

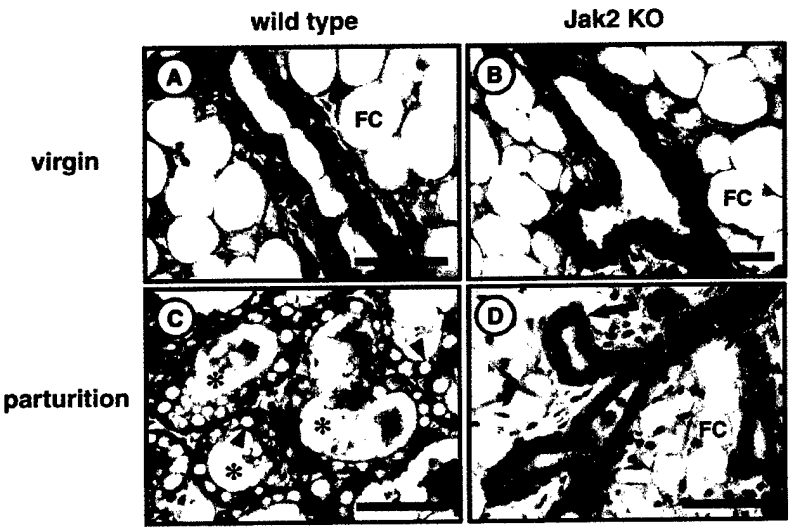

Fig. 2. Jak2 Is Required for Functional Differentiation of the Mammary Epithelium

The whole mounts in Fig. 1 were embedded in paraffin, sectioned and processed for hematoxylin and eosin staining. No apparent differences in overall epithelial development are observed when comparing virgin wild-type (A) with Jak2-null (B) transplanted epithelium at 8 wk. Alveolar development in wild-type (C) and Jak2-null (D) transplanted epithelium at parturition. Alveoli with large, expanded lumen and luminal secretory products are evident in the wild-type epithelium (C, *asterisks*). Lipid droplets are also apparent in the secretory cells (C, *arrowheads*). No lipid synthesis is observed in Jak2-null epithelium (D), and the presence of a putative nonsecretory, alveoli-like structure is indicated (D, *arrow*). FC, Fat cell. *Bar*, 50 μM.

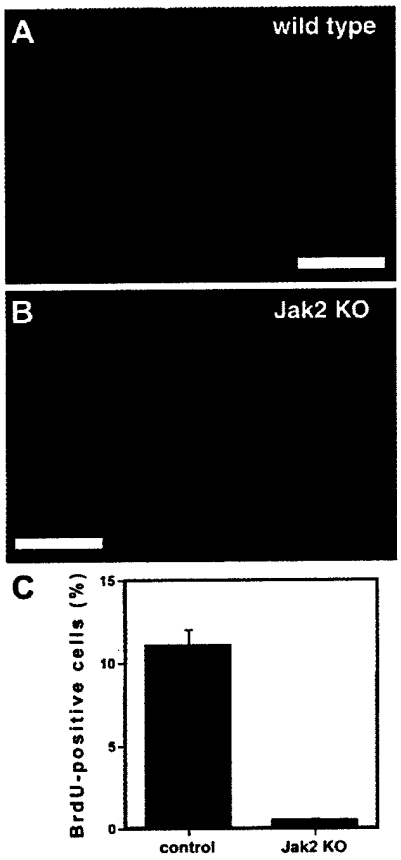

Fig. 3. Reduced Proliferation of Epithelial cells in the Absence of Jak2

Transplanted mice were injected with estrogen and progesterone (E + P). After E + P treatment, mice were injected with BrdU and the glands harvested and subsequently fixed. Incorporation of BrdU was detected using a FITC-conjugated anti-BrdU antibody (green) and nuclei were visualized with DAPI (*blue*). BrdU localization in (A) wild-type and (B) Jak2-null epithelium showed far fewer BrdU-positive cells in Jak2-null epithelium. C, Counts revealed a 96% reduction in BrdU-positive cells in Jak2-null (0.47% ± 0.15) *vs.* wild-type (11.26% ± 0.9) epithelium (mean ± SEM). It should be noted that the majority of ducts in the Jak2-null samples were BrdU-negative and the image shown in (B) depicts a rare proliferating cell. *Bar*, 50 μM.

Maintenance of a Ductal Cell Marker and Lack of a Secretory Cell Marker in Jak2-Null Epithelium at Parturition

It was clear from the whole mount and histological analyses that the absence of Jak2 resulted in a failure of mammary epithelium to develop into distinct alveolar structures at parturition. Furthermore, Jak2-null transplants at pregnancy d 13 were also underdeveloped and were comparable to epithelial development seen in Jak2-null transplants at parturition (data not shown).

To investigate whether Jak2-null epithelium at parturition retained ductal characteristics and failed to acquire features characteristic of secretory epithelium, we investigated the expression of markers preferentially expressed in ductal or secretory epithelial cells. We have established that the Na-K-Cl cotransporter, NKCC1, is present at high levels on the basolateral membrane of ductal epithelial cells during virgin mammary gland development (Fig. 4A; and Shillingford, J. M., K. Miyashi, M. Flagella, G. E. Shull, and L. Henninghausen, unpublished data). During pregnancy (data not shown) and lactation (Fig. 4C) the levels of NKCC1 in wild-type alveoli are much reduced and only a few cells within the ducts maintain high levels of NKCC1 (data not shown). By EST database searches we also determined that an epithelial Na-Pi cotransporter, Npt2b (20), was preferentially expressed in cDNA libraries derived from lactating but not from nonlactating mammary tissue (data not shown). Therefore, these data suggest that NKCC1 and Npt2b serve as markers for ductal and secretory epithelial cells, respectively.

Jak2-null epithelium isolated from virgin mice (Fig. 4B) showed equivalent levels of NKCC1 protein as wild-type virgins (Fig. 4A). However, while a reduction in NKCC1 protein was observed in wild-type epithelium at pregnancy (data not shown) and parturition (Fig. 4C), Jak2-null epithelium maintained high levels of NKCC1 protein at parturition (Fig. 4D) similar to wild-type virgin epithelium (Fig. 4A). A comparable maintenance of NKCC1 expression was observed in transplanted Stat5- and PrlR-null epithelium at parturition (7). Taken together, the observed persistence of NKCC1 protein in Jak2-null epithelium at parturition demonstrates that the absence of Jak2 results in a lack of differentiation of the mammary epithelium during pregnancy and the retention of ductal features.

We further examined the expression of Npt2b. In wild-type glands, Npt2b protein was not detectable in ductal epithelium in virgin tissue (Fig. 4E) or alveolar structures at midpregnancy (data not shown). However, at late pregnancy (d 18) Npt2b was detected on the apical membrane of a few secretory alveoli (data not shown) and on the apical membrane of all alveoli at parturition (Fig. 4G, *arrowheads*). These results suggest that Npt2b is a marker of secretory cell function. Similar to virgin wild-type epithelium, ductal epithelium in Jak2-null virgin transplants showed no evidence of apical Npt2b (Fig. 4F). However, in contrast to wild-type epithelium at parturition, apical Npt2b was not detectable in Jak2-null epithelium at parturition (Fig. 4H). Based on maintenance of a high level of basolateral NKCC1 protein and lack of apical Npt2b protein, we conclude that Jak2-null mammary epithelium retains ductal characteristics and fails to acquire a marker indicative of secretory function.

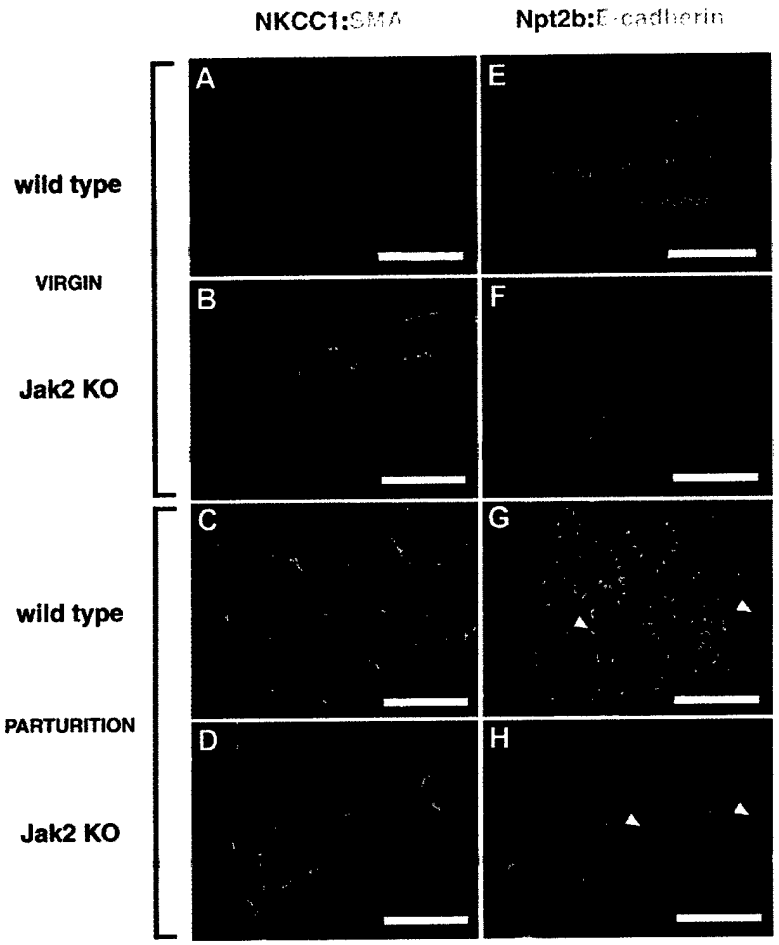

Fig. 4. Maintenance of a Ductal Cell Marker and Lack of a Secretory Cell Marker in Jak2-Null Epithelium

Transplanted tissue was harvested from virgin mice and mice 1 d after parturition. After fixation, tissue was embedded in paraffin, sectioned, and subsequently stained with specific antibodies. Localization of NKCC1 (*red*) and smooth muscle actin (SMA, *green*) in (A) virgin wild-type ductal epithelium, (B) Jak2-null virgin epithelium, (C) wild-type epithelium at parturition, and (D) Jak2-null epithelium at parturition. Note the persistence of a high level of NKCC1 protein in Jak2-null epithelium at parturition (D) similar to virgin wild-type ductal epithelium (A). In contrast, much reduced levels of NKCC1 are observed in wild-type alveoli at parturition (C). Localization of Npt2b (*red*) and E-cadherin (*green*) in (E) virgin wild-type ductal epithelium, (F) virgin Jak2-null epithelium, (G) wild-type epithelium at parturition and (H) Jak2-null epithelium at parturition. Npt2b protein is evident on the apical membrane of wild-type alveoli at parturition (G, *arrowheads*) but is notably absent in Jak2-null epithelium at parturition (H, *arrowheads*). No detectable Npt2b is apparent in virgin wild-type (E) or Jak2-null epithelium (F). *Bar*, 50 μM.

Nuclear Translocation of Stat5a in the Absence of Jak2

It is well established that phosphorylation of Stat5 induces Stat5 dimerization, which in turn results in subsequent Stat5 nuclear translocation (21). In the context of the mammary gland PRL activates Stat5 via the PrlR and its associated kinase, Jak2, and the absence of either the PrlR or Stat5 inhibits functional differentiation of the mammary epithelium at pregnancy (7). Therefore, we examined the localization of Stat5a in wild-type and Jak2-null epithelium isolated from mice at pregnancy d 13 (Fig. 5A and B, respectively) and at parturition (Fig. 5, C and D, respectively). In wild-type glands at pregnancy d 13 (Fig. 5A), nuclear Stat5a staining was observed (*arrowheads*). In contrast, many fewer cells exhibited nuclear Stat5a in Jak2-null epithelium (Fig. 5B, *arrowheads*), and a large number of cells had no detectable Stat5a (*arrows*). After parturition, nuclear Stat5a was apparent in almost every epithelial cell in wild-type glands (Fig. 5C, *arrowheads*). Similar to that seen in pregnancy, Jak2-null epithelium at parturition (Fig. 5D) showed evidence of a few cells with nuclear Stat5a (*arrowheads*), cells with cytoplasmic Stat5a, and cells with no detectable Stat5a (*arrows*).

To quantitate Stat5a nuclear localization, the number of Stat5a-positive nuclei were counted and expressed as a percentage of the total number of luminal epithelial cells (Table 1). Counting of two independent

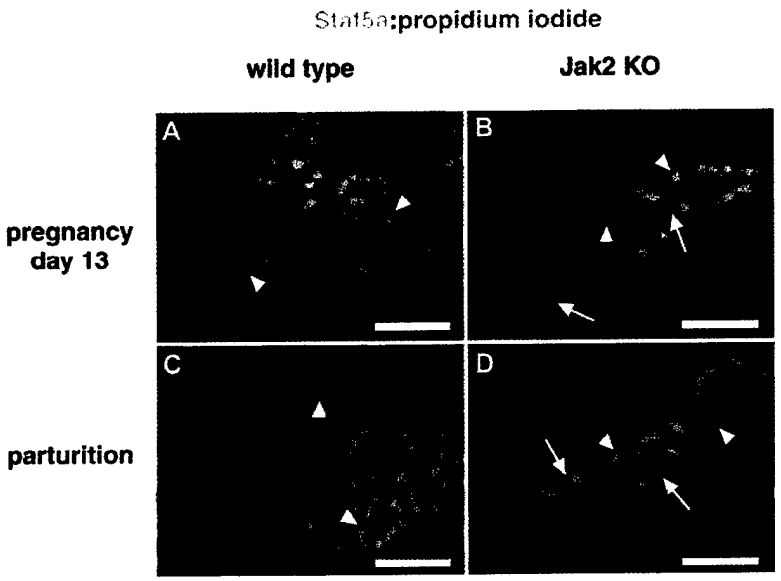

Fig. 5. Nuclear Localization of Stat5a in the Absence of Jak2

Wild-type (A and C) and Jak2-null (B and D) mammary transplants were harvested at pregnancy d 13 (A and B) and at parturition (C and D). Nuclei were stained with propidium iodide (*red*). Significant nuclear Stat5a (*green*) staining was apparent in wild-type glands at pregnancy d 13 (A, *arrowheads*), but less so in Jak2-null epithelium (B, *arrowheads*), which contained a proportion of cells with no detectable Stat5a (B, *arrows*). At parturition, most of the wild-type alveoli contained nuclear Stat5a (C, *arrowheads*) whereas only a few Jak2-null cells showed evidence of Stat5a nuclear staining (D, *arrowheads*). Note that some cells lacked detectable Stat5a staining (D, arrows). *Bar*, 50 μM.

Table 1. Nuclear Stat5a *vs.* Total Number of Luminal Epithelial Cells

	Jak2-null	Wild-type
Pregnancy d 13 A	26.8 ± 2.1%	71.2 ± 1.5%
Pregnancy d 13 B	23.1 ± 1.1%	74.8 ± 2.8%
Parturition A	19.6 ± 1.4%	79.7 ± 2.1%
Parturition B	25.7 ± 2.0%	79.9 ± 2.2%

samples revealed a highly significant difference ($P < 0.0001$) between Stat5a-positive nuclei in Jak2-null cells *vs.* wild-type cells at pregnancy d 13 (25% *vs.* 73%, respectively) and at parturition (23% *vs.* 80%, respectively). The observed nuclear localization of Stat5a in the absence of Jak2 could be mediated by a number of alternative signaling pathways independent of Jak2 function, including activation of phosphatidylinositol-3-kinase (22, 23), c-Src (24, 25), MAPK (10, 26) and signaling pathways activated by the intrinsic kinase activity of the epidermal growth factor receptor (4). However, despite evidence of limited Stat5a nuclear translocation in Jak2-null epithelium, these cells fail to proliferate and differentiate to form functional secretory tissue. A possible reason for this is that a threshold of activated Stat5 may be required to enable cells to switch from a ductal cell lineage to an alveolar cell lineage, which is not achieved in the absence of Jak2. Such a threshold could be perceived as 1) the number of cells expressing nuclear Stat5 and/or 2) the absolute amount of Stat5a in the nucleus. Unfortunately, an accurate quantitation of the absolute amount of nuclear Stat5, which would further address this important question, is not readily attainable. Despite this, the data presented herein provide strong experimental evidence that in the absence of Jak2, other signaling pathways do not play a major role in the proliferation or differentiation of the mammary gland. This is the first *in vivo* study that defines Jak2 as a critical mediator of proliferative and differentiation events in the mammary epithelium at pregnancy.

MATERIALS AND METHODS

Antibodies

The NKCC1 and Npt2b antibodies were gifts from Dr. Jim Turner, NIDCR, NIH (Bethesda, MD) and Dr. Jurg Biber, University of Zurich (Zurich, Switzerland), respectively. The E-cadherin and Stat5a (L-20) antibodies were obtained from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively.

Experimental Animals

All animals used in the course of this study were treated within published guidelines of humane animal care.

Mammary Gland Transplantation

This technique has been described previously (27). Briefly, *Jak2* hemizygous mice in a pure C57BL/6 genetic back-

ground (14) were mated and euthanized at d 12.5 of pregnancy. Embryos were removed and identified visually for the loss of *Jak2*, as assessed by the lack of erythropoesis in the liver, and confirmed by PCR as previously described (14). Embryonic mammary glands were dissected from the *Jak2*-null, hemizygous and wild-type embryos and cultured overnight. The endogenous mammary epithelium from the no. 4 gland of 3-wk-old athymic (nu/nu) mice was removed, and the embryonic mammary cultures were placed into the remaining epithelial-free fat pad. Routinely, Jak2-null and Jak2 wild-type glands were contralaterally transplanted in the same mouse. Mice were left for 8 wk before isolation of virgin mammary tissue, or mating and mammary tissue harvest during pregnancy or at parturition. For some experiments, successful outgrowths were transplanted a second time into nude mice as previously described (28). Whole mount analyses were performed as described previously (29).

Immunofluorescence Analyses

Isolated tissue was fixed in Tellyesniczky's fixative for 4 h at room temperature and embedded in paraffin by routine methods. Sections were boiled in an antigen unmasking solution (Vector Laboratories, Inc., Burlingame, CA) for 2 min followed by 10 min incubation. Primary antibodies were applied (NKCC1, 1:1000; Npt2b, 1:100; E-cadherin, 1:200; Stat5a 1:200) and the sections incubated for 1 h at 37 C (NKCC1, Npt2b and E-cadherin) or overnight at room temperature (Stat5a). Sections were incubated with fluorescent-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature in the dark. Mounting medium (Vectashield, Vector Laboratories, Inc.) was applied, and the sections were analyzed. Immunofluorescence was viewed under a Carl Zeiss Axioscop (Carl Zeiss, Inc., Thornwood, NY) equipped with filters for FITC (fluorescein isothiocyanate), TRITC (tetramethyl rhodamine isothiocyanate) and FITC:TRITC. For Stat5a nuclear counts, the total numbers of luminal epithelial cells *vs.* nuclear Stat5a positive cells were counted in ten separate fields at 63× magnification (63× objective plus 10× eyepiece = 630×) from two independent mice. Statistical significance was assessed using *t* test.

Cell Proliferation Analyses

For proliferation experiments, virgin mice 9 wk after transplantation were treated for 48 h with 1 μg β-E2 (Sigma, St. Louis, MO) and 1 mg progesterone (Sigma) in 100 μl sesame oil via a single interscapular sc injection. Two hours before they were killed, mice were injected with 0.3 mg BrdU per 10 g body weight (Amersham Pharmacia Biotech, Arlington Heights, IL), and both of the transplanted number 4 inguinal mammary glands and an endogenous number 3 gland (control) were removed. Isolated tissue was fixed in 4% paraformaldehyde in PBS for 2 h at 4 C. BrdU-positive cells were detected by immunofluorescence as described previously (30). BrdU-positive cells and total nuclei were counted from 16 fields at 60× magnification (60× objective plus 10× eyepiece = 600×). Statistical analysis was performed using a two-tailed *t* test.

Acknowledgments

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Address all correspondence and requests for reprints to: Dr. Jonathan Shillingford, Laboratory of Genetics and Physiology, National Institutes of Health, 9000 Rockville Pike, Room 105, Building 8, Bethesda, Maryland 20817. E-mail: jonshi@helix.nih.gov.

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Article

Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium

Keiko Miyoshi,[1,5] Jonathan M. Shillingford,[1] Gilbert H. Smith,[2] Sandra L. Grimm,[3] Kay-Uwe Wagner,[1,4] Takami Oka,[1] Jeffrey M. Rosen,[3] Gertraud W. Robinson,[1] and Lothar Hennighausen[1]

[1]Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, and [2]Laboratory of Tumor Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892
[3]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030
[4]University of Nebraska Medical Center, Omaha, NE 68198
[5]Department of Biochemistry, School of Dentistry, The University of Tokushima, Tokushima, Japan 770-8504

Functional development of mammary epithelium during pregnancy depends on prolactin signaling. However, the underlying molecular and cellular events are not fully understood. We examined the specific contributions of the prolactin receptor (PrlR) and the signal transducers and activators of transcription 5a and 5b (referred to as Stat5) in the formation and differentiation of mammary alveolar epithelium. PrlR- and Stat5-null mammary epithelia were transplanted into wild-type hosts, and pregnancy-mediated development was investigated at a histological and molecular level. Stat5-null mammary epithelium developed ducts but failed to form alveoli, and no milk protein gene expression was observed. In contrast, PrlR-null epithelium formed alveoli-like structures with small open lumina. Electron microscopy revealed undifferentiated features of organelles and a perturbation of cell–cell contacts in PrlR- and Stat5-null epithelia. Expression of NKCC1, an Na-K-Cl cotransporter characteristic for ductal epithelia, and ZO-1, a protein associated with tight junction, were maintained in the alveoli-like structures of PrlR- and Stat5-null epithelia. In contrast, the Na-Pi cotransporter Npt2b, and the gap junction component connexin 32, usually expressed in secretory epithelia, were undetectable in PrlR- and Stat5-null mice. These data demonstrate that signaling via the PrlR and Stat5 is critical for the proliferation and differentiation of mammary alveoli during pregnancy.

Introduction

Cytokines, such as prolactin (Prl),* growth hormone (GH), interleukin (IL)-2, and erythropoietin (Epo) elicit a wide range of cell-specific responses including cell proliferation, survival, differentiation, and death. Upon binding of these cytokines to their respective receptors, the receptor-associated kinase Jak2 phosphorylates the latent transcription factors signal transducer and activator of transcription (Stat)5a and Stat5b at tyrosines 694/699, respectively. Upon activation, Stat5a and Stat5b form homo- and heterodimers that translocate to the nucleus and induce cell-specific genetic programs. In erythroid cells, Epo-induced Stat5 activation may lead to the transcriptional activation of the *bcl-x* gene and thus promote cell survival (Socolovsky et al., 1999), and in T cells, IL-2–mediated cell proliferation is severely impaired in the absence of Stat5 (Teglund et al., 1998; Moriggl et al., 1999).

Development of the mammary gland occurs predominantly in the postnatal animal and is controlled by steroid and peptide hormones (Hennighausen and Robinson, 1998, 2001). Proliferation and differentiation of mammary alveolar epithelia occurs during pregnancy through prolactin and its receptor (PrlR) (Ormandy et al., 1997b). Stat5a-null mice fail to develop functional mammary tissue during pregnancy, mainly as a result of impaired functional differentiation and not due to the lack of lobulo-alveolar units (Liu et al., 1997). After multiple pregnancies, functional mammary

Address correspondence to Keiko Miyoshi, Laboratory of Genetics and Physiology NIDDK, National Institutes of Health Bldg. 8, Rm. 101, Bethesda, MD 20892-0822. Tel.: (301) 496-2716. Fax: (301) 480 7312. E-mail: mammary@nih.gov

*Abbreviations used in this paper: Epo, erythropoietin; GH, growth hormone; IL, interleukin; PI3K, phosphoinositide 3-kinase; PrlR, prolactin receptor; RT, reverse transcription; Stat, signal transducer and activator of transcription; WAP, whey acidic protein; ZO, zonula occludens.

Key words: prolactin receptor; Stat5; mammary gland; cell specification; epithelia

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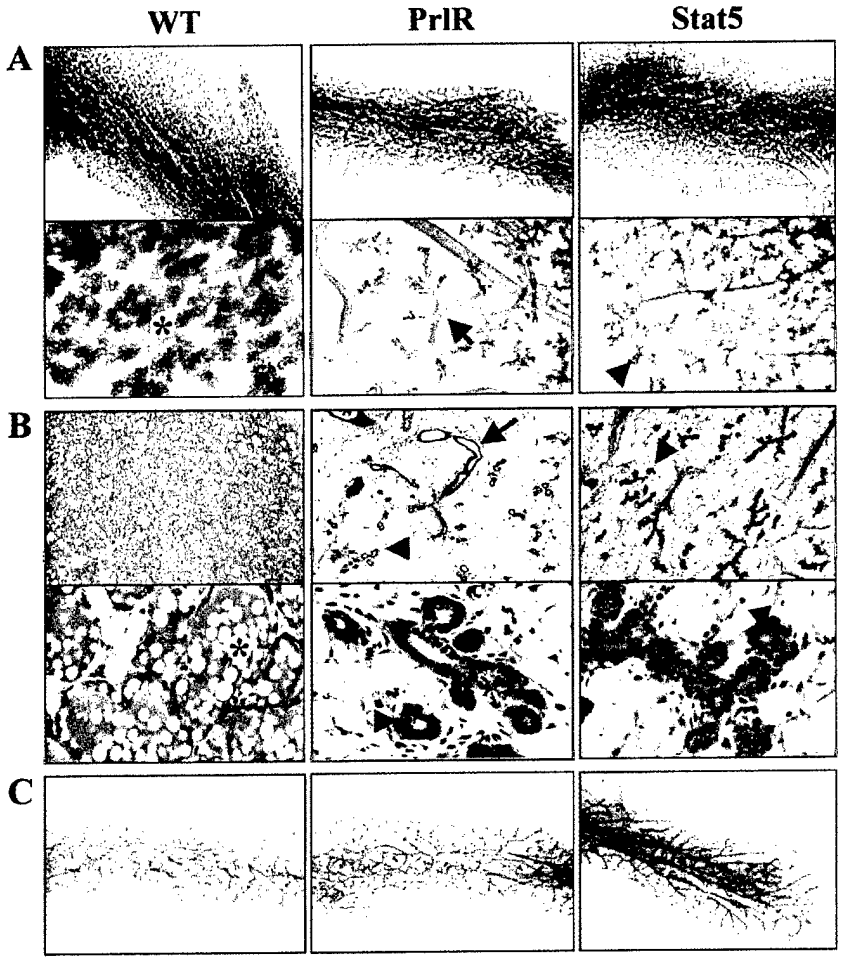

Figure 1. **Pregnancy-mediated mammary gland development depends on the PrlR and Stat5.** (A) Whole mount analyses of wild-type, PrlR-, and Stat5-null mammary epithelia at parturition. The lower panel represents a higher magnification. Wild-type epithelium filled the fat pad and developed lobuloalveolar structures (*). PrlR- and Stat5-null mammary epithelia were severely underdeveloped. PrlR-null epithelium exhibited wide ducts (black arrow), short branches, and few alveoli-like structures. Stat5-null epithelium displayed normal branches with small decorations (black arrowhead). (B) Histological sections of the whole mounts shown in A. The lower panel represents a 6× higher magnification. Wild-type epithelium was fully expanded, filled the fat pad and alveolar lumina contained milk (*). However, all null epithelia were sparse, and the alveoli-like structures (black arrowhead) did not contain milk. The epithelial cells are cuboidal in shape (white arrow). Note the presence of open lumina in the alveoli-like structures in PrlR-, but not Stat5-null epithelium. (C) Whole mount analyses of wild-type and PrlR- and Stat5-null virgin mammary epithelia 8 wk after transplantation. All transplanted epithelia completely filled the fat pad.

development was attained in Stat5a-null mice (Liu et al., 1998). This phenotype was accompanied by increased levels of active Stat5b, suggesting that Stat5b can partially compensate for the absence of Stat5a. However, Stat5b itself is not required for lactation (Teglund et al., 1998). Because mice carrying inactivated Stat5a and 5b (referred to as Stat5 throughout the text) genes are infertile, the combined function of both Stat5a and 5b during pregnancy had not been investigated.

Components of regulatory pathways are in many cases not exclusive, and may participate in several signaling cascades. In mammary epithelia, Stat5 is activated through the PrlR, but also by GH and the epidermal growth factor receptors (Gallego et al., 2001), and possibly the *Src* pathway (Kazansky et al., 1999). In addition, the PrlR not only activates Stat5 via Jak2, but also stimulates the mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) pathways (Bole-Feysot et al., 1998; Kim and Cochran, 2001). If mammary alveolar epithelial development depends on a pathway that includes the PrlR and Stat5, a similar phenotype should be expected in the respective gene deletion mice. However, if Prl-independent Stat5 activation controls some steps of development, a different phenotype would be expected in the absence of the two components (i.e., PrlR and Stat5).

We have now investigated the relative contributions of Stat5 and the PrlR in pregnancy-induced mammary epithelial development. Although gross morphological analyses have linked the PrlR (Ormandy et al., 1997a) and Stat5a (Liu et al., 1997) to cell proliferation and differentiation, the molecular and cellular mechanisms of prolactin and Stat5 signaling are not understood. Specifically, the combined roles of Stat5a and 5b during pregnancy-induced mammopoiesis are not known. It remains unclear whether the Prl pathway is actually required for the acquisition of a particular cell fate, or whether it controls the differentiation of an already specified cell type. We have used molecular markers that can distinguish between different mammary epithelial cell types in the developing mammary gland to investigate the role of PrlR and Stat5 in the development of alveolar epithelium.

Results

Development of mammary tissue in PrlR- and Stat5-null mice

Although the PrlR is required for development of mammary epithelium during pregnancy (Ormandy et al., 1997b), the contribution of Stat5 in this process is not known. Stat5 is downstream of the PrlR, and it can be hypothesized that the loss of either signaling component might lead to a comparable phenotype. To investigate this, we compared the pregnancy-induced development of PrlR- and Stat5-null epithelia. Because Stat5- and PrlR-null mice are infertile,

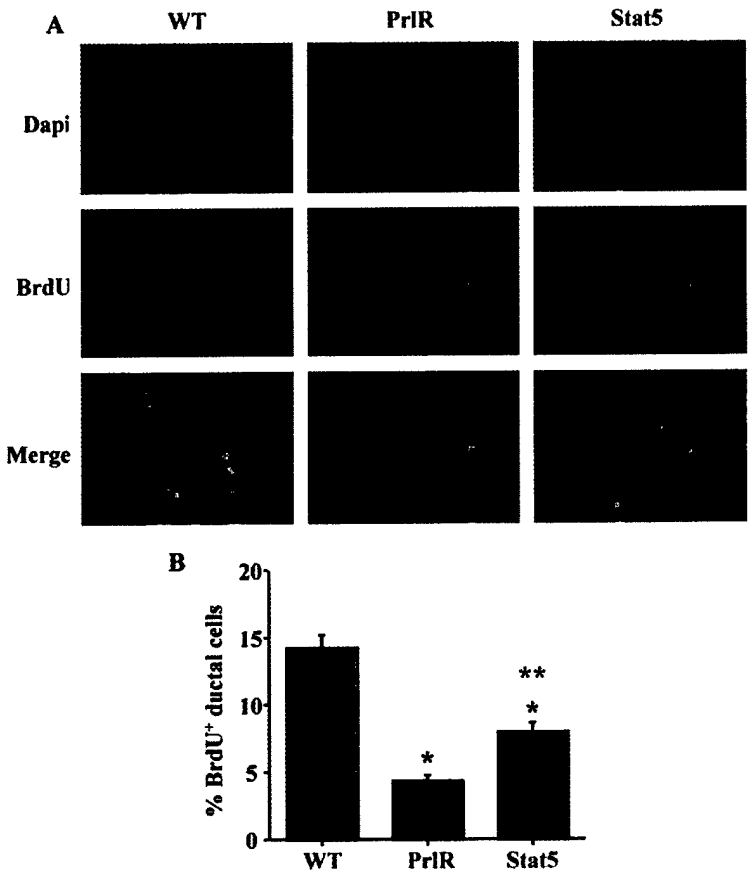

Figure 2. **Progesterone- and estrogen-induced proliferation is more severely impaired in PrlR-null than in Stat5a/b–null mammary epithelium.** 9 wk after transplantation, mice were given an acute, 2-d E+P treatment. Mammary glands were removed and proliferation was evaluated by BrdU immunostaining. (A) Green staining represents BrdU-positive cells and DAPI-stained nuclei are blue. Merging the images shows the proliferation occurring mainly in the ductal epithelium. The number of BrdU-positive cells was clearly decreased in both PrlR- and Stat5-null transplants, as compared with the control #3 gland. (B) Quantitation of BrdU-positive ductal cells (mean percentage ± SEM) is shown in the bar graph. The *P* values comparing PrlR- or Stat5-null to control (*) and Stat5-null to PrlR-null (**) were <0.001 as determined by Mann-Whitney paired *t* test.

mammary epithelia from mature virgins was transplanted into the cleared fat pad of recipients to expose these epithelia to pregnancy hormones. Whole mount analyses demonstrated that ductal development during puberty was not affected in PrlR- and Stat5-null mice (Fig. 1 C). In contrast, pregnancy-mediated alveolar development was severely impaired (Fig. 1 A). Whereas wild-type ducts were decorated with expanded alveoli, the epithelium was greatly reduced in Stat5-null transplants and did not have the appearance of true lobulo-alveolar units. Histological sections demonstrated that the majority of Stat5-null alveoli-like structures did not have lumina (Fig. 1 B, right panel). Furthermore, individual Stat5-null epithelial cells exhibited abnormal columnar shapes and the epithelial architecture appeared disorganized (Fig. 1 B, right panel, white arrow). On the other hand, small open lumina were evident in the more organized alveoli-like structures in the PrlR-null transplants (Fig. 1 B, middle panel). To further evaluate the differences between Stat5- and PrlR-null alveoli-like structures, we analyzed serial sections. Whereas PrlR-null tissue exhibited consistently open lumina, they were not apparent in Stat5-null tissue (unpublished data).

Proliferation of PrlR- and Stat5-null mammary epithelia

We investigated potential causes for the lack of functional alveolar development in PrlR- and Stat5-null epithelia, and examined estrogen- and progesterone-mediated proliferation (Fig. 2). Previous studies have demonstrated that acute treatment with estrogen and progesterone for 2 d resulted in ~15% of the wild-type ductal epithelial cells entering the cell cycle, as assessed by BrdU labeling (Seagroves et al., 2000). Maximal proliferation appears to occur in the alveolar progenitors in the ducts during the first few days of pregnancy. The estrogen and progesterone treatment of virgin transplants that have filled the fat pad is designed to mimic the effect of early pregnancy on alveolar development. In comparison to the thoracic #3 controls from the host animal, steroid hormone–induced proliferation of PrlR-null epithelium was reduced by ~60%, whereas proliferation of Stat5-null epithelium was reduced by only 30% (Fig. 2 B). Thus, whereas in both cases there was a significant reduction in proliferation relative to the wild-type control, PrlR-null epithelium exhibited a twofold greater decrease than Stat5-null epithelium. These differences were statistically significant ($P < 0.001$).

Differentiation of PrlR- and Stat5-null mammary epithelia

There were no morphological and histological signs of milk secretion in PrlR- and Stat5-null epithelia (Fig. 1 B). To determine the differentiation status of PrlR- and Stat5-null epithelia, we examined the expression of milk protein genes (β-casein, whey acidic protein [WAP], and WDNM1). Steady-state levels of these mRNAs increased in wild-type (Fig. 3) and Stat5b-null (unpublished data) mammary tissue during pregnancy. Expression of WAP, but not β-casein, mRNA was decreased in Stat5a-null mice (Fig. 3)

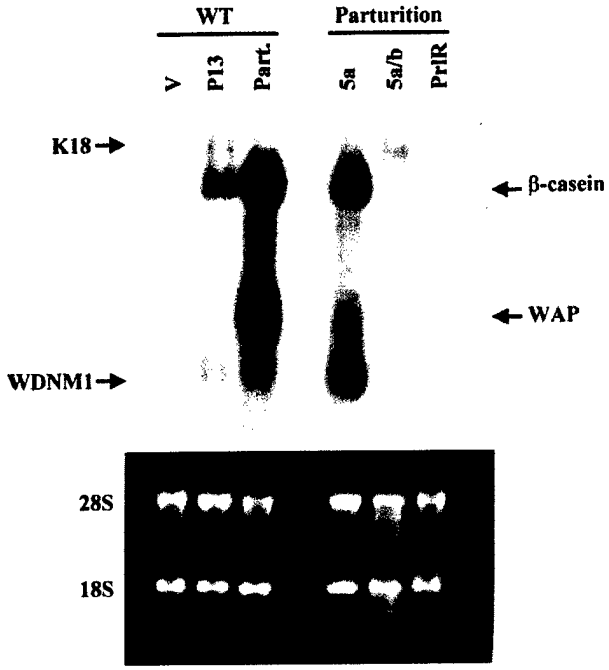

Figure 3. **Milk protein expression is impaired in Stat5- and PrlR-null mammary epithelia.** Northern blot analysis of milk protein mRNAs (β-casein, WAP, WDNM1) and keratin 18 (K18) mRNA in mammary tissue of wild-type mice (WT) and Stat5a-, 5a/b-, and PrlR-null epithelial transplants. V, virgin (5 wk); P13, pregnancy day 13; Part, after parturition.

as shown previously (Liu et al., 1997). β-casein and WAP mRNAs were not detected in PrlR- and Stat5-null epithelia by total RNA Northern blot. On the other hand, WDNM1 mRNA was detected at lower levels in the PrlR- and Stat5-null epithelia. Using more sensitive assays, low levels of WAP and β-casein were detected in Stat5-null epithelia (unpublished data). These results suggest that PrlR- and Stat5-null epithelia failed to undergo functional differentiation.

Stat5-null epithelium have impaired cell–cell contacts and lack connexin 32

We evaluated the ultrastructure of wild-type, Stat5-, and PrlR-null mammary epithelia at parturition by electron microscopy (Fig. 4). Mammary tissue from lactating control mice contained secretory epithelia with features indicative of a fully differentiated phenotype: the alveolar lumina were expanded and contained casein micelles and lipid droplets (Fig. 4, A and D). Alveolar cells contained a well-developed Golgi apparatus and a rough endoplasmic reticulum (Fig. 4 D). In contrast, PrlR- (Fig. 4, B and E) and Stat5-null (Fig. 4, C and F) epithelial structures were highly disorganized. Most of the alveoli-like structures in Stat5-null epithelium had no lumina (Fig. 4, C and F). Occasionally, the lumina that were present that were small, and did not contain casein micelles and lipid droplets (Fig. 4 F). Often, there were several layers of epithelia so that the lumina seemed congested with cells (Fig. 4 C). We frequently observed several pseudo-lumina within one structure, suggesting the inability to form a single lumen surrounded by a single layer of epithelial cells (unpublished data). Interestingly, the cells near the basement membrane, thought to be myoepithelia, contained lipid droplets reminiscent of secretory cells (Fig. 4 C). In contrast, PrlR-null epithelia were more organized and the alveoli-like structures contained small but open lumina (Fig. 4, B and E). Neither the rough endoplasmic reticulum nor

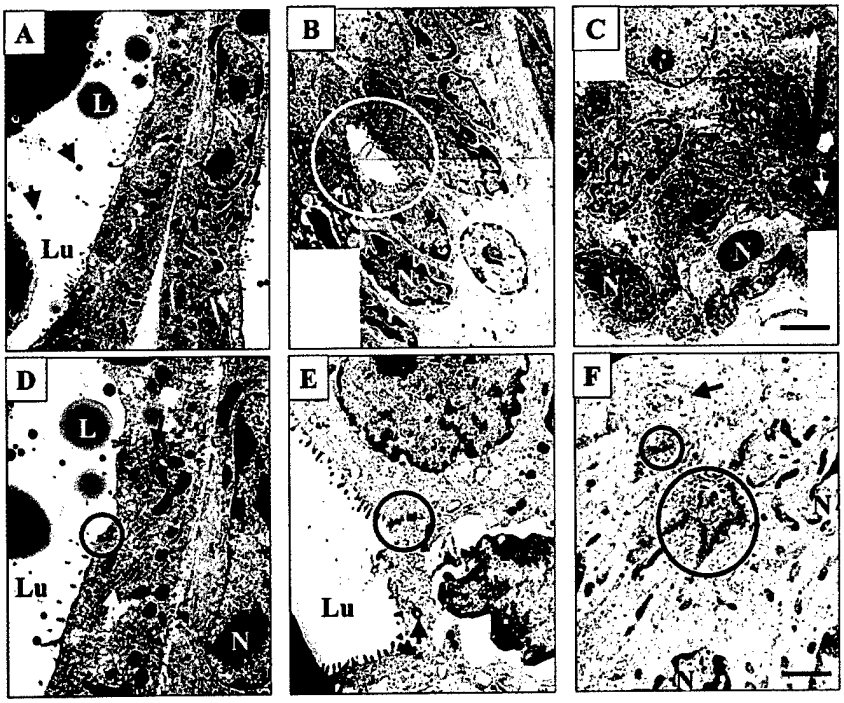

Figure 4. **Disorganized structures of Stat5- and PrlR-null epithelia.** Stat5- and PrlR-null mammary epithelia at parturition were analyzed by electron microscopy. (A and D) Control mammary epithelium at lactation day 1 was fully differentiated and contained secreted milk proteins and lipid droplets. Golgi apparatus (white long arrow) and RER (white long arrow) were detected. Lu, lumen; L, lipid droplet; N, nucleus; black short arrow, β-casein micelles; black circle, tight junction. (B and E) Alveoli-like structures of PrlR-null epithelium were more organized than Stat5-null epithelia. Lumina were detected in alveolar-like structure (white circle). The centrosome was located close to the apical membrane (black arrow). Tight junctions were maintained (black circle). (C and F) Alveoli-like structures in Stat5-null epithelia were disorganized and cell–cell contacts were aberrant (black arrow). Frequently, two or more pseudo-lumina were detected in one alveolar-like structure (black circles). The cells near the basement membrane contained lipid droplet-like structures (white arrow). Active Golgi apparatus and a RER in Stat5- and PrlR-null epithelia were not apparent. Bars: (A–C) 2.4 μm; (D–F) 1.6 μm.

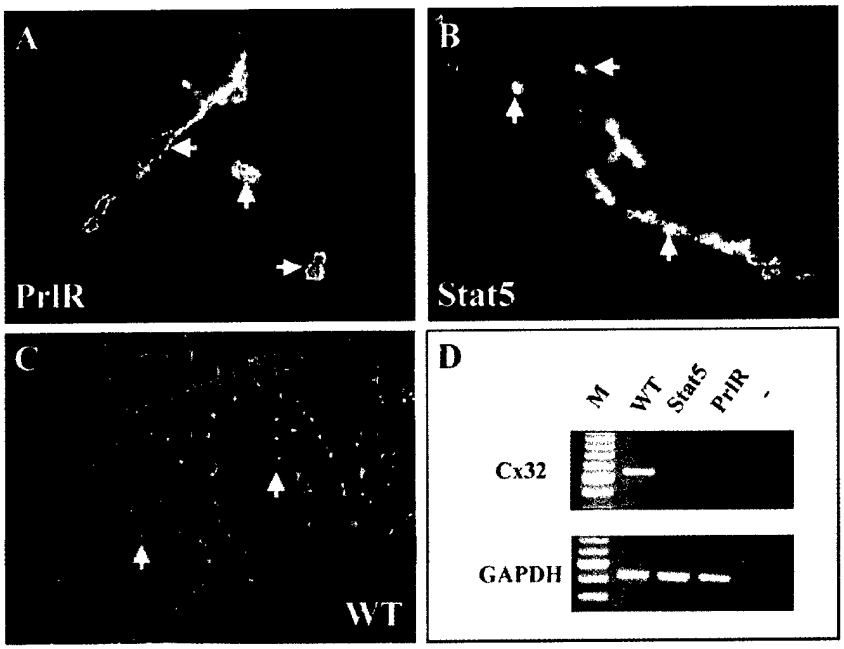

Figure 5. **Maintenance of ZO-1 expression but loss of Cx 32 expression.** Immunohistochemical staining of ZO-1 (green) and E-cadherin (red) at parturition (A–C). Tight junctions (green dots) were present in PrlR- (A) and Stat5-null (B) the same as in wild-type epithelium (C). Arrows point to alveoli-like structures of PrlR- and Stat5-null epithelia. (D) RT-PCR analysis of Cx 32 mRNA. Total RNA from wild-type, Stat5-, and PrlR-null transplanted epithelia at parturition was reverse transcribed, and the cDNA was subjected to PCR. Cx 32 cDNA was detected in wild-type but not in PrlR- or Stat5-null samples. GAPDH levels were similar in all samples. M, marker.

the active Golgi apparatus were prominent in PrlR- or Stat5-null epithelial cells (Fig. 4, E and F). We observed that the epithelial cells furthest from the basement membrane, and therefore the most "luminal," contained centrosomes located close to their apical membrane as determined by the presence of microvilli (Fig. 4 E). This centrosomal orientation suggested that these cells had recently divided and that their cleavage plane was perpendicular to the basement membrane potentially resulting in the epithelial layering and the crowding of the luminal space.

Epithelial cells contact each other via tight and adherens junctions (Cereijido et al., 1998; Borrmann et al., 2000; Vasioukhin and Fuchs, 2001), which stabilize epithelial structures and determine their integrity. Further, these junctions are necessary to establish and maintain cell polarity (Knust, 2000; Vicente-Manzanares and Sanchez-Madrid, 2000) that permits vectorial secretion (Barcellos-Hoff et al., 1989). Fig. 4 D shows the morphological appearance of a tight junction complex between the apical poles of two individual secretory cells in wild-type epithelium. One of the most conspicuous features of the Stat5-null epithelium was the lack of organized cell contacts. To identify possible causes for the lack of organized cell contacts we investigated the expression of zonula occludens (ZO)-1, a component of tight junctions. Tight junctions were identified in PrlR- and Stat5-null epithelia (Fig. 4, E and F) and visualized by ZO-1 staining (Fig. 5, A and B). We further investigated the expression of connexin, a protein in the gap junction complex, in PrlR- and Stat5-null epithelia. It has been demonstrated previously (Pozzi et al., 1995; Locke et al., 2000) that mouse mammary tissue expresses three connexin isoforms (Cx 43, Cx 26, and Cx 32), which we confirmed using reverse transcription (RT)-PCR analyses. Whereas Cx 32 mRNA was detected in wild-type epithelia at day 1 of lactation, we were unable to detect expression in PrlR- and Stat5-null epithelia at parturition (Fig. 5 D).

To further examine the cell adhesion defect apparent in Stat5-null epithelial cells, we investigated the expression of additional molecules involved in cell adhesion. Cadherins mediate cell–cell adhesion and also contribute to the maintenance of apical–basal polarity (Tepass et al., 2000). Indeed, E-cadherin has been shown to play a role in the morphogenesis and growth of the mammary gland (Daniel et al., 1995; Delmas et al., 1999). On this basis, we hypothesized that E-cadherin expression might be altered in Stat5-null mammary epithelial cells and thus contribute to the observed alterations in cell adhesion. However, E-cadherin expression along cell–cell borders did not appear to be significantly perturbed when comparing Stat5-null and wild-type epithelia (Figs. 5 and 7).

PrlR- and Stat5-null epithelia maintain virgin-like ductal features during pregnancy

Whole mount and histological analyses have demonstrated that the formation of mammary alveolar epithelium was severely impaired in the absence of the PrlR or Stat5. However, these types of analyses do not allow the identification of the epithelial cells as ductal or alveolar. To further determine the histological identity of these cells, we investigated the expression of proteins that characterize either ductal or secretory alveolar epithelium. We have observed that the Na-K-Cl cotransporter (NKCCl) is expressed at high levels in virgin mice and is located on the basolateral membrane of ductal epithelial cells, and unpublished data). Further, NKCC1 expression levels decreased in developing alveoli but are maintained in some cells of the ductal epithelia during pregnancy. Therefore, we examined the expression of NKCC1 by immunohistochemistry in transplanted PrlR- and Stat5-null epithelia in virgin mice and at parturition. In virgin mice, NKCC1 was detected in endogenous epithelium and PrlR- and Stat5-null transplants (Fig. 6, A, C, and E). At parturition, the levels of NKCC1 were sharply re-

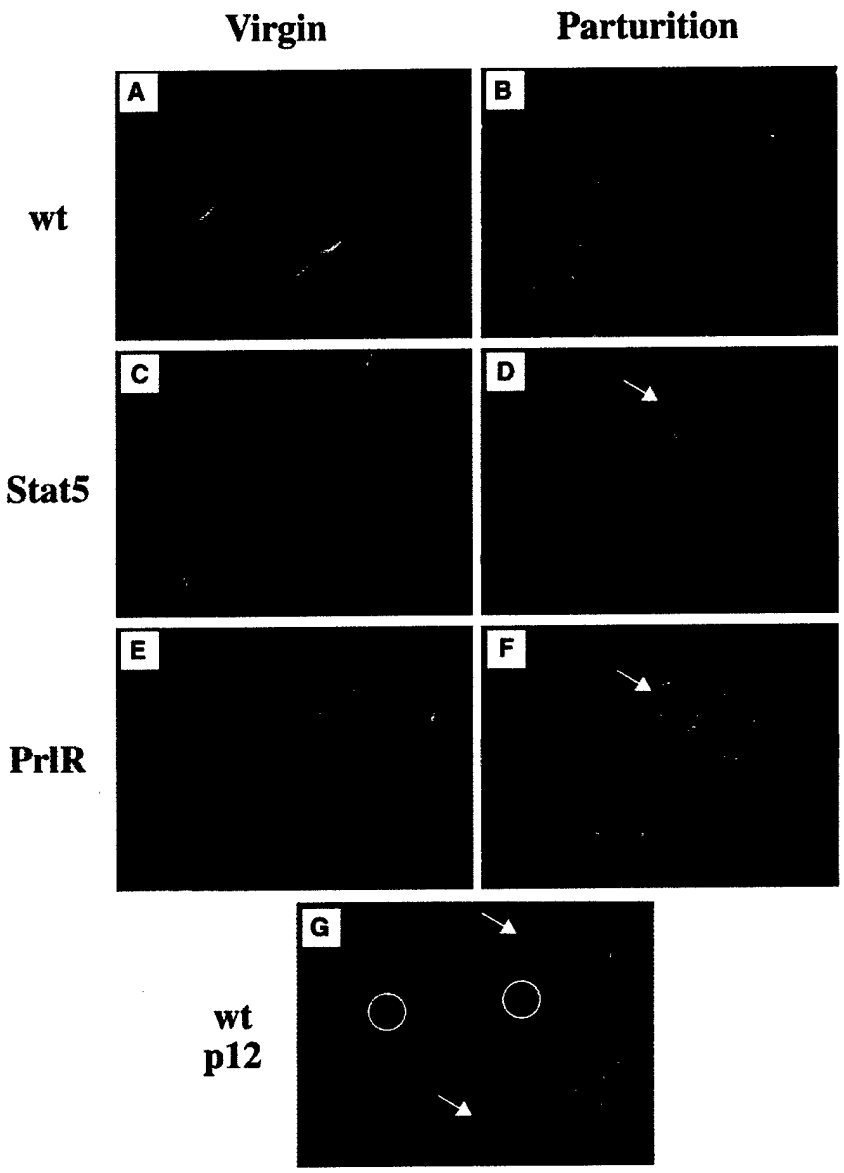

Figure 6. **NKCC1 and smooth muscle actin are expressed in PrlR- and Stat5-null alveoli-like structures at parturition, but not in wild-type mice.** Immunohistochemical staining of NKCC1 (red) and smooth muscle actin (green) in mammary epithelia of virgin mice (A, C, and E) and after parturition (B, D, and F). NKCC1 levels were high in ductal epithelium of wild-type virgin mice (A) and sharply reduced in alveoli by pregnancy day 12 (G, circle) and at parturition (B). PrlR- and Stat5-null epithelia maintained high levels of NKCC1 at parturition (D and F, compare white arrows with white arrow in G).

duced in wild-type secretory alveolar cells (Fig. 6 B). In contrast, NKCC1 expression was consistently higher in PrlR- and Stat5-null epithelia at parturition (Fig. 6, D and F), and the alveoli-like structures stained strongly for NKCC1. At pregnant day 12, wild-type alveolar cells already had reduced NKCC1 levels (Fig. 6 G). These results suggest that the cells forming alveoli-like structures still maintain features of ductal epithelia. Smooth muscle actin, which characterizes myoepithelial cells, was found in PrlR- and Stat5-null, and control epithelia from virgin mice (Fig. 6, A, C, and E). At parturition, smooth muscle actin staining demonstrated a contiguous layer of thin myoepithelial cells surrounding the fully expanded wild-type alveoli (Fig. 6 B). On the other hand, PrlR- and Stat5-null (Fig. 6, D and F) epithelia exhibited a staining pattern very similar to that seen in virgin wild-type epithelium (Fig. 6 A). The expression pattern of smooth muscle actin in alveoli-like structures was identical to that observed in ducts (Fig. 6, A, E, and F).

In an attempt to identify potential markers of secretory function, we searched the mouse EST database for genes preferentially expressed in the lactating mammary library, NMLMG. Using this approach, we discovered that a sodium phosphate cotransporter isoform, Npt2b, was highly expressed in the mouse mammary gland library derived from lactating tissue, but not virgin tissue (unpublished data). Next, we examined the expression of Npt2b during normal mammary gland development. RT-PCR analyses demonstrated that Npt2b mRNA was not present in virgin or early (day 13) pregnancy, but was evident during late (day 16) pregnancy through mid (day 5) lactation (unpublished data). The cellular localization of Npt2b protein was addressed by immunohistochemistry. We detected expression of Npt2b protein in mammary tissue from day 18 pregnant mice and at day one of lactation (Fig. 7), but not in virgin or early pregnant mice (unpublished data). Interestingly, Npt2b protein was ex-

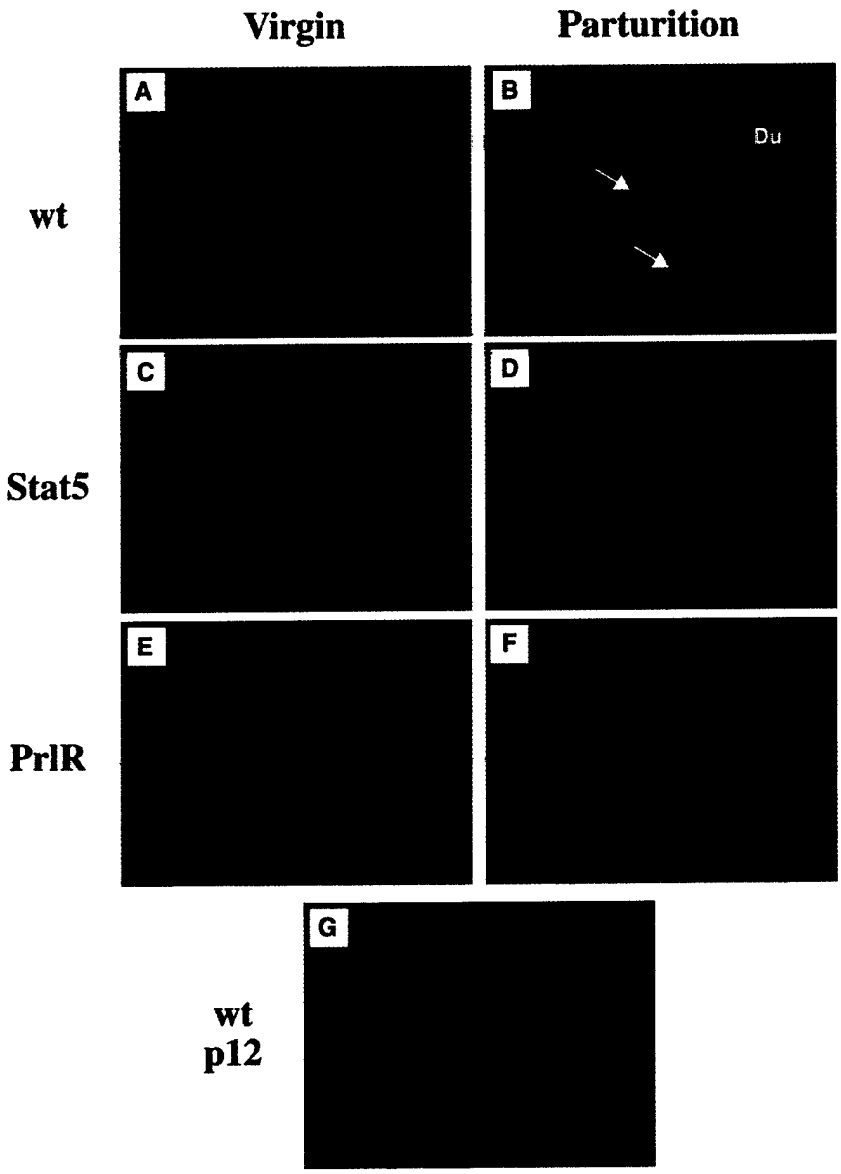

Figure 7. **Npt2b expression was not detected in PrlR- and Stat5-null alveoli-like structures at parturition.** Immunohistochemical staining of Npt2b (red) and E-cadherin (green) in mammary epithelia of virgin mice (A, C, and E) and after parturition (B, D, and F). Npt2b was detected in apical membranes of secreting epithelia from wild-type tissue (B, white arrow). PrlR- and Stat5-null epithelia at parturition did not contain Npt2b (D and F). E-cadherin was detected in the sub-apical/basolateral membrane of all samples (A–F). (G) Wild-type epithelia at pregnancy day 12 did not express Npt2b. Du, duct.

pressed on the apical membrane of the mammary secretory cells (unpublished data). Taken together, these results suggest that Npt2b is a potential marker of secretory function. As mentioned above, PrlR- and Stat5-null epithelia fail to secrete milk proteins suggesting they were devoid of normal secretory function. Therefore, we examined the expression of Npt2b protein in these samples (Fig. 7). Whereas the mammary secretory cells present in wild-type epithelia showed expression of Npt2b in the apical membrane (Fig. 7 D), Npt2b was not detected in PrlR- and Stat5-null epithelia (Fig. 7, E and F). In contrast, E-cadherin (Fig. 7) was expressed in the basolateral membrane of all samples examined.

Epidermal growth factor and GH can activate Stat5 in PrlR-null epithelium

Based on histological and electron microscopy studies, Stat5- and PrlR-null epithelia exhibited differences. Whereas Stat5-null epithelium was highly disorganized and did not form open lumina, PrlR-null epithelium formed small open lumina. The observation that Stat5-null epithelium exhibited a more severe phenotype than PrlR-null epithelium, suggested that Stat5 might be activated to some extent by other cytokines in the absence of the PrlR. We have recently demonstrated by Western blot analysis that EGF and GH can activate Stat5 in mammary tissue (Gallego et al., 2001). However, their respective receptors within the epithelial compartment are not required for functional development (Gallego et al., 2001). We now investigated whether Stat5 could be activated in PrlR-null epithelium using immunohistochemistry (Fig. 8). Because Stat5a is more abundant than Stat5b, and Stat5b is not critical for alveolar development, we examined Stat5a activation. Stat5-null epithelia served as a negative control. At parturition, Stat5a was localized within nuclei of wild-type alveolar and ductal

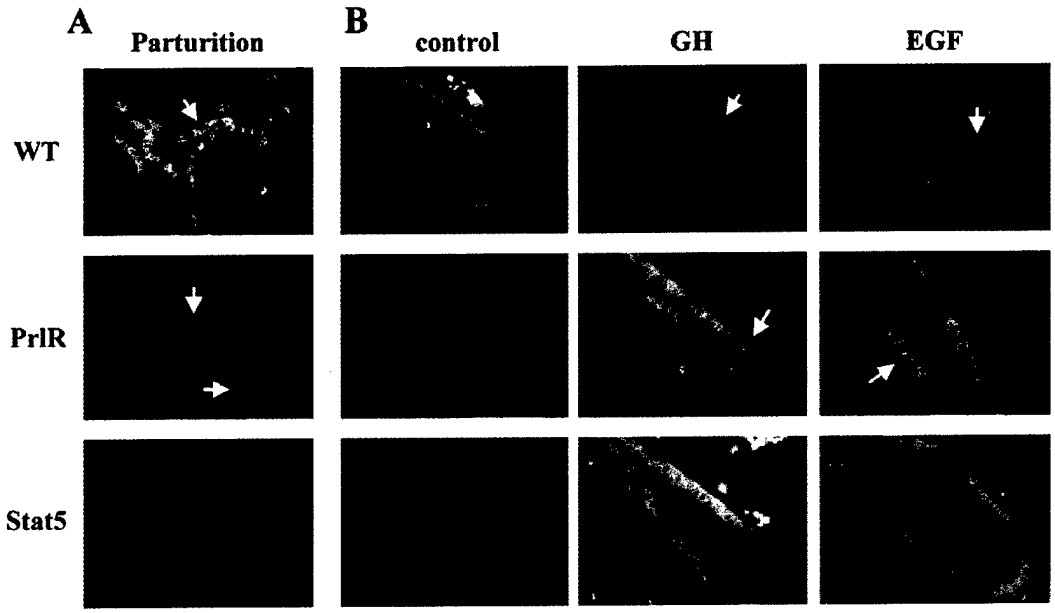

Figure 8. **Epidermal growth factor and GH can activate Stat5a in PrlR-null epithelium.** Immunohistochemical staining of Stat5a (green) and E-cadherin (red) in mammary epithelium at parturition (A) and in virgin tissue after hormone injection (B). Arrows show Stat5a nuclear staining. (A) In PrlR-null epithelium, some Stat5a nuclear staining was detected. In contrast, almost all cells in wild-type epithelia had Stat5a nuclear translocation. (B) Virgin mice carrying PrlR- and Stat5-null epithelia were injected with EGF or GH. Extensive nuclear localization of Stat5a was observed in PrlR-null epithelium but not in Stat5-null epithelium.

cells, which is indicative of its active state (Fig. 8 A). In contrast, only a few cells in PrlR-null epithelia exhibited nuclear, and thus activated Stat5a. However, upon injection with EGF or GH, extensive nuclear translocation of Stat5 was observed in PrlR-null epithelium (Fig. 8 B) indicative of Prl-independent activation of Stat5a.

Discussion

Here we demonstrate that Stat5 controls the establishment of functionally differentiated and secreting alveoli during pregnancy (Fig. 9). This pathway is activated to a large extent, but not exclusively, through the PrlR. We propose that activation of the Stat5 pathway by prolactin, GH and epidermal growth factor transduces signals that instruct cells at the branch points to proliferate and adopt alveolar characteristics. Further, we suggest that Stat5 determines cell fate through the establishment of cell–cell adhesion.

The PrlR–Stat5 pathway is obligatory for mammary alveolar cell fate

The transcription factor Stat5 is central to several signaling pathways, and is activated by various cytokines through their respective receptors and Jak2 (Ihle, 2001). Because Stat5-null mice are infertile (Teglund et al., 1998), their mammary development had not been studied during pregnancy. We avoided the problem of infertility through the transplantation of knockout mammary epithelia into wild-type hosts. Although a ductal tree developed in the absence of Stat5, no alveolar development was apparent after one pregnancy. Whereas Stat5-null mammary epithelium exhibited a similar phenotype, it was distinct from that observed in the absence of the PrlR (Fig. 9). Ductal branching occurred in the absence of Stat5, but the alveoli-like structures did not form lumina as were observed in the absence of the PrlR. Our results suggest that Stat5 plays a role in regulating cell organization through cell–cell contacts independent of PrlR activation. Based on molecular markers that characterize ductal epithelia and secretory alveolar epithelia, we suggest that the PrlR- and Stat5-null alveoli-like structures have retained ductal-like characteristics. Thus, we propose that an early function of the prolactin pathway in mammary epithelia is the specification and determination of alveolar epithelia. Two separate and distinct lineage-limited mammary epithelial progenitors have been identified in the mouse mammary gland (Kordon and Smith, 1998; Smith, 1996). The failure to develop secretory alveoli may be either due to a failure to generate the alveoli-limited progenitor or the inability of the alveoli-limited progenitor and its progeny to respond to prolactin signals. It is apparent that proliferation of ductal epithelia can occur in the absence of a functional PrlR–Stat5 pathway. However, inactivation of the PrlR or Stat5 results in the formation of small alveoli-like structures with ductal features that we propose to name "ductoli." Even after several pregnancies, we did not detect functional alveoli in PrlR- and Stat5-null transplants (unpublished data). This suggests that if compensatory pathways are activated, they are unable to elicit functional alveolar development.

In addition to the mammary gland phenotype observed in the transplant studies described herein, the Stat5-null mice exhibit phenotypes in other tissues and cell types consistent with the inactivation of several cytokine signaling pathways (Teglund et al., 1998). Loss of Stat5 disrupts IL-2 signaling and results in impaired T cell proliferation and a failure to express

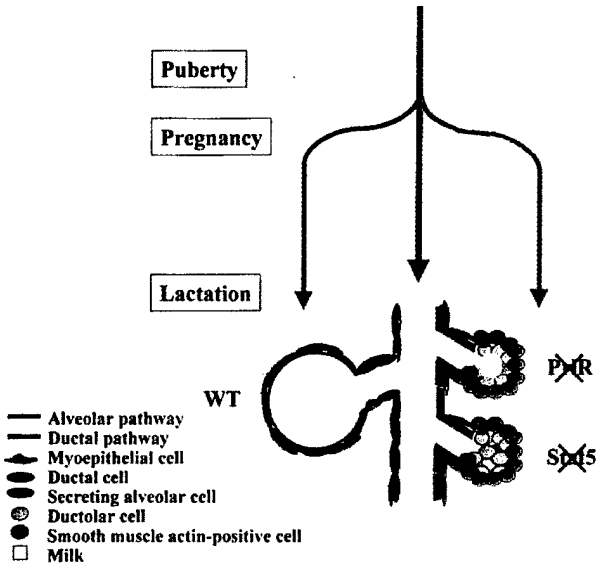

Figure 9. **PrlR and Stat5 control the cell fate of mammary alveolar epithelium.** Wild-type mammary epithelium differentiates into functional alveoli during pregnancy. However, PrlR- and Stat5-null epithelia do not undergo alveolar development. The null epithelia maintain ductal features and cell proliferation at the branching points results in the development of "ductoli" (ductal feature but alveoli-like structure). In contrast to Stat5-null ductoli, PrlR-null ductoli contain open lumina. In the absence of Stat5 ductolar cells have impaired cell–cell contacts. The ductoli do not differentiate into functional alveoli. Blue line, ductal development; red line, alveolar development. The myoepithelial cells surrounding the alveoli are flat. The outer cell layer of ductoli is positive for smooth muscle actin and can thus be considered to be of myoepithelial nature.

genes controlling cell cycle progression (Moriggl et al., 1999). In addition, Stat5 has also been linked to cell survival (Humphreys and Hennighausen, 1999; Socolovsky et al., 1999; Schwaller et al., 2000; Ihle, 2001). Lastly, Stat5 has been linked to B cell differentiation induced by IL-4 and IL-7 (Sexl et al., 2000) and Stat5a is required for functional differentiation, but not proliferation, of mammary epithelial cells (Liu et al., 1997).

Cell–cell adhesion defects in PrlR- and Stat5-null mammary epithelia

The absence of Stat5 resulted in defective cell–cell adhesion as assessed by electron microscopy. Thus, in control samples the basolateral membranes of neighboring cells were in close contact with each other. Conversely, there was evidence of gaps between adjacent cells in the Stat5-null samples. This suggests that Stat5 mediates signals that promote cell–cell adhesion. The expression of E-cadherin and ZO-1, proteins known to be involved in cell–cell adhesion and associated with tight junctions, respectively (Gumbiner, 1996), revealed normal staining patterns in both the PrlR- and Stat5-null samples. However, it is possible that whereas adjacent cells express detectable E-cadherin, they may not physically contact each other. In addition to E-cadherin, the process of cell adhesion involves additional proteins and it cannot be ruled out that any one of these are responsible for the cell adhesion defects that we observed.

The establishment of cell–cell adhesion is not only important for epithelial organization, but also for effective communication between individual cells. Such intercellular communication allows neighboring cells within a defined structural unit to respond in unison to a given signal. For example, the gap junction subunit Cx 43 is expressed in the myoepithelial cell compartment, and it has been suggested that this may help coordinate myoepithelial contraction and milk ejection upon oxytocin stimulation (Plum et al., 2000). The lack of appropriate gap junction protein expression, and by extension intercellular communication, could disrupt the cellular unit (i.e., alveoli) whose formation may be required for their full functional development. It has previously been shown that the mouse mammary gland expresses three connexin isoforms, Cx 26, Cx 32, and Cx 43 (Pozzi et al., 1995). Whereas Cx 43 is expressed in myoepithelial cells, both Cx 26 and Cx 32 are expressed in the epithelial compartment. Interestingly, Cx 32 expression is induced at lactation and cannot readily be detected at other time points, suggesting that it may contribute to the attainment of a secretory phenotype (Locke et al., 2000). Furthermore, Cx 32 has been shown to interact with proteins in the tight junction complex and determine cell polarization in hepatocytes (Kojima et al., 2001). Although we were able to detect Cx 32 expression in control samples at parturition, neither PrlR- nor Stat5-null epithelia expressed detectable levels of Cx 32. It is possible that the lack of Cx 32 expression in PrlR- and Stat5-null epithelia is the result of a lack of secretory differentiation. Alternatively, it is possible that Cx 32 is a Stat5 target gene. In fact, the mouse Cx 32 gene promoter contains an interferon γ–activated sequence site at position –800, suggesting that this promoter may be under direct prolactin control.

We have provided experimental evidence on the level of histology and electron microscopy that cell-cell adhesion and organization is impaired in the absence of Stat5, and to a lesser extent in the absence of the PrlR. Although we have shown apparent normal localization of E-cadherin and ZO-1, this cannot be a true measure for their functional integrity, which needs to be addressed in future studies. There is now widespread interest in the regulation of mammary epithelial cells by cell–cell adhesion molecules. It is likely that many proteins, including the transcription factor Stat5, control these processes.

Pathway redundancy

There were notable differences when comparing the individual phenotypes on the histological level. In particular, epithelial development in the absence of the PrlR was more inhibited than in the absence of Stat5. This could in part be explained by a reduction in the proliferative capacity of PrlR-null epithelium relative to Stat5-null epithelium. Such differences may be due to the activation of compensatory signaling pathways. For example, mitogen-activated protein kinase and PI3K can be activated upon Prl stimulation. It is also possible that other Stats (i.e., Stat1 and/or Stat3) may be recruited to Jak2 in the absence of Stat5, thus resulting in additional stimulation of epithelial development.

There was evidence of open lumina in the PrlR-, but not in the Stat5-null, transplants. Furthermore, the ductoli

present in the PrlR-null epithelia were more organized compared with Stat5-null epithelia, suggesting that Stat5 is necessary for appropriate organization of individual cells into cohesive cellular structures. Whereas Prl is probably the key cytokine responsible for the activation of Stat5, we demonstrated that Stat5 has some residual activity in the absence of the PrlR. Because both GH and EGF activate Stat5 in the absence of the PrlR, it is likely that these two cytokines contribute to the formation of cell–cell contacts and thus a lumen.

Pathways controlling alveolar epithelial development

We have established that the PrlR and Stat5 are each essential for the attainment of functional alveologenesis. Several other genes and signaling pathways that control alveolar development have been identified, including ErbB2 (Jones and Stern, 1999) and ErbB4 (Jones et al., 1999), cyclin D1 (Fantl et al., 1995; Sicinski et al., 1995), C/EBPβ (Robinson et al., 1998; Seagroves et al., 1998), the osteoclast differentiation factor RANKL and its receptor RANK (Fata et al., 2000), and the helix-loop-helix protein Id2 (Mori et al., 2000). Data from these mouse models suggests that alveologenesis is a complex process requiring the functional cooperation of numerous molecules. Interestingly, comparable phenotypes were observed in some of these mice, i.e., lack of alveolar development. Developmental roles for ErbB2 and 4 have been suggested based on transgenic mice that express dominant negative forms under control of a mouse mamary tumor virus long terminal repeat. Expression of a dominant negative ErbB2 resulted in condensed alveoli and reduced luminal secretion at parturition (Jones and Stern, 1999). ErbB4-dominant negative epithelium formed condensed alveoli and failed to expand at mid lactation, which correlated with reduce expression of α-lactalbumin and WAP and a loss of Stat5 activity (Jones et al., 1999).

Similar to the models described here, C/EBPβ-null mice possess undifferentiated alveolar epithelium; in contrast, branching morphogenesis was also impaired. C/EBPβ mRNA levels in PrlR- and Stat5-null transplanted epithelia at parturition were similar to those seen in wild-type tissue (unpublished). These results suggest that C/EBPβ expression is essentially independent of the PrlR–Stat5 pathway, although they may converge at the β-casein promoter (Wyszomierski and Rosen, 2001). In the absence of RANKL, a growth factor produced by mammary epithelia in the second half of pregnancy, mammary epithelia also fail to develop (Fata et al., 2000). Because Prl can activate RANKL expression (Fata et al., 2000), it may be downstream of Stat5. However, both RANKL and RANK are expressed at high levels in PrlR- and Stat5-null transplanted epithelia at parturition (unpublished data), suggesting that these pathways are parallel and not dependent on each other. Id2-deficient mice also show severely impaired mammary gland development (Mori et al., 2000). Furthermore, Id2-deficient mammary epithelia exhibit reduced phosphorylation of Stat5. Normal levels of Id2 mRNA were detected in PrlR- and Stat5-null epithelia at parturition (unpublished data), suggesting that Id-2 is not downstream of Stat5. The presence of several, and apparently parallel, pathways controlling mammary alveolar development further emphasizes that distinct signals contribute to alveologenesis. At this point it is not clear whether these pathways have unique molecular targets leading to the formation of functional alveoli. The understanding of signaling pathways that are required for the formation of mammary epithelia but are dispensable for life of the organism itself provides a unique opportunity to develop molecular interventions and prevention for breast cancer.

Materials and methods

Animals

The Stat5a/b–null mice (Teglund et al., 1998) were bred into the C57BL/6 background. PrlR-null mice (Ormandy et al., 1997b) were in a C57BL/6 background, and Stat5a-null mice (Liu et al., 1997) were in a C57BL/6 and 129 mixed background. For the transplantation studies of PrlR- and Stat5-null mammary epithelia, athymic NCr-nu/nu mice were used as hosts. Wild-type littermates were used as controls. More than 30 PrlR- and Stat5-null transplants each were analyzed. For hormone injection studies, the transplanted virgin mice (12 wk after transplantation) were used. The mice were injected by intraperitoneally with either murine GH (5 μg/g of body weight), or human EGF (10 μg/g of body weight). Mammary glands were harvested 15 min later, fixed in 4% paraformaldehyde for 4 h, and processed for paraffin embedding and sectioning by standard procedures.

Antibodies

Polyclonal anti–rabbit Stat5a antibodies have been described previously (Liu et al., 1996). Mouse monoclonal E-cadherin and smooth muscle actin antibodies were obtained from Transduction Laboratories, polyclonal anti–rabbit ZO-1 antibodies were purchased from Zymed Laboratories, and polyclonal anti–rabbit NKCC1 antibodies (Moore-Hoon and Turner, 1998) were a gift from Dr. Jim Turner (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD). The polyclonal anti–rabbit Npt2b antibodies (Hilfiker et al., 1998) were a gift from Dr. Jurg Biber (Department of Physiology, University of Zurich, Zurich, Switzerland).

Transplantation of adult mammary epithelia into the cleared fat pad of nude mice

The transplantation was performed as previously described (DeOme et al., 1959). In brief, small pieces of mammary tissue were excised from mature virgin female wild-type, Stat5-, or PrlR-null mice. Athymic nude mice (3-wk-old) were anesthetized with an intraperitoneally injection of avertin and the proximal part of the inguinal gland containing the mammary epithelium was excised. Pieces of mammary tissue from a PrlR- and a Stat5-null mouse were grafted into contralateral cleared fat pads of recipients. The other combinations of transplants were Stat5:wild type and PrlR:wild type. To assess the completeness of clearing, the excised endogenous glands were processed for whole mount staining according to standard protocols. 8 wk after transplantation, fat pads containing transplants were harvested from virgin hosts. Alternatively, the hosts were bred and tissue was harvested on the day of parturition. For whole mounts, mammary glands were removed, fixed in Carnoy's fixative overnight and stained in carmine alum.

Immunofluorescence

After fixation in Tellyesniczky's fixative for 4 h at room temperature, tissues were embedded in paraffin and sectioned at 5 μm. Sections were cleared in xylene and rehydrated. Antigen retrieval was performed by heat treatment using an antigen unmasking solution (Vector Laboratories) and tissue sections were blocked for 30 min in PBST containing 10% goat or horse serum. For ZO-1 detection, antigen retrieval was performed by protease treatment (Auto/Zyme Reagent set; Biomeda Corp.) at 37°C for 10 min. Sections were incubated with E-cadherin (1:1,000) and ZO-1 (1:500) antibodies, E-cadherin (1:1,000) and Npt2b (1:100) antibodies, smooth muscle actin (1:1,000) and NKCC1 (1:1,000) antibodies or E-cadherin (1:1,000), and Stat5a (1:250) antibodies. The primary antibodies were allowed to bind for 60 min at 37°C except for Stat5a:E-cadherin and ZO-1:E-cadherin (4°C, overnight). Nonspecifically bound antibody was removed by rinsing in PBST before the addition of both anti–mouse FITC-conjugated (1:250) and anti–rabbit Texas red–conjugated (1:250) secondary antibodies. Sections were incubated in the dark for 30 min, washed in two changes of PBST, and mounted in Vectashield (Vector Laboratories, Inc.). Fluorescence was visualized with a Zeiss Axioscop microscope equipped with FITC, TRITC, and FITC:TRITC filters. Images were captured using a Sony DKC5000 digital camera.

Analysis of cellular proliferation

After 9 wk, the transplant recipients were treated for 48 h with 1 μg β-estradiol (E) (Sigma-Aldrich) and 1 mg progesterone (P) (Sigma-Aldrich) in 100 μl sesame oil via a single interscapular subcutaneous injection behind the neck. After acute hormone treatment, both of the transplanted number 4 inguinal mammary glands and an endogenous number 3 gland (control) were removed. 2 h before sacrifice, mice were injected with 0.3 mg BrdU per 10 g body weight (Amersham Pharmacia Biotech). Tissue was fixed in 4% paraformaldehyde in PBS for 2 h at 4°C. Immunofluorescence and BrdU-positive cell counting were performed as previously described (Seagroves et al., 2000). In brief, paraffin sections (5–7 μm) were dewaxed and subjected to microwave antigen retrieval in 10 mM citrate buffer, pH 6.0. After blocking in 5% BSA/0.5% Tween 20 for 4 h at room temperature, sections were incubated with anti–BrdU-FITC–conjugated antibody (1:5; Becton Dickinson) in blocking solution overnight at room temperature. After PBS washes, slides were mounted in Vectashield + DAPI medium (Vector Laboratories). At least four glands per genotype were used for each experiment (endogenous control #3, $n = 5$; PrlR-null epithelia transplanted gland, $n = 5$; Stat5-null epithelia transplanted gland, $n = 4$). Cells from 16 fields at 60× magnification were counted from each sample. The number of BrdU-positive cells in a given field was expressed as a percentage of the total number of DAPI-stained cells. Statistical significance was determined by Mann-Whitney paired *t* test.

Gene expression analysis

Total RNA was isolated from fresh or frozen tissues and Northern blots were performed as described previously (Robinson et al., 1995). In brief, 10 μg of total RNA was loaded in each lane. Membranes were hybridized with random-primed [α-^{32}P] dCTP-labeled probes in QuickHyb solution for 3 h at 65°C. Washes were performed in 0.1 × SSC/0.1% SDS at 65°C. At first, the membrane was hybridized with WAP and β-casein probes together and exposed to x-ray films. After stripping, membranes were rehybridized with WDNM1 and keratin 18 probes together. A 415-bp WAP-specific probe was generated by RT-PCR with primers 5′-GTA-CCA-TGC-GTT-GCC-TCA-TC-3′ and 5′-GCT-GCT-CAC-TGA-AGG-GTT-ATC-3′. A 577-bp β-casein–specific probe was generated by RT-PCR with primers 5′-CTA-AAG-TTC-ACT-CCA-GCA-TCC-3′ and 5′-CAT-TTC-CAG-TTT-CAG-TCA-GTT-C-3′. A full-length cDNA for WDNM1 was used (Robinson et al., 1995). The keratin 18–specific probe was a 1.1-kb EcoRI cDNA fragment (Singer et al., 1986).

Electron microscopy

Small pieces of mammary tissue were cut, minced into 1-mm cubes, and fixed in a 0.025% solution of glutaraldehyde and 3.8% paraformaldehyde in PBS, pH 7.2, for 2 h. The tissue was rinsed in PBS and post-fixed in 2% osmium tetroxide in 0.5 M sodium cacodylate buffer for 2 h, and dehydrated in a graded series of acetone solutions (Blanchette-Mackie and Scow, 1971). Temperature was maintained at 4°C from excision through dehydration and tissues were embedded in epon at room temperature (Luft, 1961). Sections were cut on a Reichert Om U2 ultramicrotome. Thick sections were stained with Toluidine blue in 1% sodium borate (pH 8.3) (Trump et al., 1961). Thin sections were stained with Karnovsky's lead hydroxide (Karnovsky, 1961) and uranyl acetate (Zobel and Beer, 1961) and examined with a JEOL 1010 electron microscope.

RT-PCR assays

Total RNA (1 μg) was transcribed into cDNA using Thermoscript reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's protocol. Total RNA (1 μg) was first incubated with dNTPs and an oligo-$dT_{(12-18)}$ primer at 65°C for 5 min. All components were added except the reverse transcriptase, and the reaction was incubated at 42°C for 2 min. Thermoscript RT (50 units) was added to each reaction and incubated for a further 50 min. For controls, the samples without RT reactions were amplified. Single-stranded RNA was degraded by treating the reaction with *Escherichia coli* RNase H for 20 min at 37°C. PCR assays were performed for Cx 32 and GAPDH cDNA. Cx 32 gene-specific primers were 5′-GTT-GCA-ACC-AGG-TGT-GGC-AGT-G-3′ and 5′-CGG-AGG-CTG-CGA-GCA-TAA-AGA-C-3′. GAPDH gene-specific primers were 5′-CAA-CGG-GAA-GGG-CCC-CCA-TAC-CAT-C-3′ and 5′-ACG-ACG-GAC-ACA-TTG-GGG-GTA-G-3′. The template was first denatured at 94°C for 2 min followed by 35 cycles (Cx 32) or 25 cycles (GAPDH) of denaturation (94°C, 40 s), annealing (65°C, 40 s), and extention (72°C, 1 min). A final extention at 72°C for 10 min was performed.

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